Gene expression associated with osteoblast differentiation

The present invention identifies genes, e.g. Hey1, whose expression pattern is altered when precursor pre-osteoblastic cells undergo differentiation into osteoblasts. The genes identified may be used as markers for the differentiation process. The present invention also provides methods to screen agents that are capable of modulating the differentiation process. The present invention also provides methods of identifying therapeutic agents that stimulate bone formation by analyzing the expression of one or more of the genes identified.

Background of the invention

Bone is a dynamic tissue in which old tissue is broken down and new tissue is synthesized. Control of the rate of breakdown and synthesis of new bone tissue is critical to the integrity of the skeletal structure. When the rates become unbalanced, serious conditions may result. The process of synthesizing new bone tissue is mediated by osteoblasts. During the process of synthesizing new bone tissue, osteoblasts differentiate from precursor pre-osteoblastic cells to mature bone-forming cells. Osteoblast differentiation process was previously examined mostly on cellular or single gene levels. Many external regulating factors are known, such as TGFb family members, but critical molecular steps in osteoblast differentiation and bone formation are largely unknown. One key player was identified recently as the transcription factor Cbfa1 (reviewed by Karsenty, 2000, Semin Cell Dev Biol; 11: 343-346). Another transcription factor, Osterix (Osx), which cooperates with and is genetically downstream of Cbfa1, has also been identified (Nakashima et al., 2002, Cell; 108:17-29). The expression levels of various enzymes and structural proteins, for example alkaline phosphatase and type-1 collagen, are up-regulated, while other genes are down-regulated. In order to treat a condition characterized by an imbalance in the rates of breakdown and synthesis of bone tissue, it may be desirable to increase or decrease the rate of break down and/or synthesis. Thus, in a number of clinical applications, it may desirable to enhance the rate of bone formation by promoting the differentiation of precursor pre-osteoblastic cells into osteoblasts. One application that is particularly important is the treatment of osteoporosis, characterized by a decrease in bone mass making the bones more fragile and subject to fracture. Other potential uses for reagents capable of affecting the synthesis of bone tissue include the healing of broken bones, recovery after surgical procedures involving bones and the like. While the changes in the expression levels of a number of individual genes have been identified, the investigation of the global changes in

gene expression, which occur in precursor stem cells as they differentiate into osteoblasts has been reported elsewhere (Qi et al., PNAS USA, 2003, 100(6), 3305-10; de Jong et al., J Bone Miner Res. 2002, 17(12): 2119-29; Vaes et al., J Bone Miner Res. 2002, 17(12):2106-18) but do not overlap (or only partially) with the finding presented in this study. However accurate information would be useful, for example, in assessing the effects of a course of treatment designed to change the rate of formation of bone tissue. Accordingly, there exists a need for the investigation of the changes in global gene expression levels, as well as the need for the identification of new molecular markers associated with the differentiation of precursor cells into osteoblasts. Furthermore, identification of additional genes involved in differentiation may allow development of reagents designed to alter their expression levels and thereby allow control of the differentiation process. In addition, identification of the genes involved in the process allows their use as diagnostic or prognostic markers which are uniquely associated with differentiation.

Bone forming osteoblasts develop from their mesenchymal precursors in vivo and in vitro. Different cellular phenotypes during osteogenesis were tentatively defined as osteoprogenitors, pre-osteoblasts, mature osteoblasts and mineralizing osteoblasts, each of them characterized by an overlapping yet distinct set of marker genes (Aubin et al., 1995, Bone; 17: 77S-83S). Cytochemically, classical markers of osteoblast differentiation are stainings for alkaline phosphatase and for mineralized bone nodules in culture. In order to examine osteoblast differentiation in vitro, a crucial step is the use of appropriate primary cells or a cell line and defined culture conditions, which allow an ordered step-wise differentiation process. This is difficult to achieve with osteosarcoma cell lines, many of which are available from human and rat origin (SaOS, U2OS, ROS, and UMR). This is because due to their tumor origin, these cell lines show de-regulated proliferation, and often express differentiation markers together with proliferation markers. In addition, these cell lines rarely exhibit formation of mineralized bone nodules in vitro, a hallmark of completed osteogenesis. In order to avoid these inherent problems with differentiation of osteosarcoma cell lines, a need for new models is imminent.

Summary of the invention

The present invention relates to the elucidation of the global changes in gene expression during osteoblastic differentiation of MC3T3-E1 cell line, in particular MC3T3-1b clone. In one aspect, the present invention relates to detecting a change in an expression level of one or more genes or gene families associated with the differentiation of MC3T3-E1

cells, in particular MC3T3-1b cells, into osteoblasts. In a related aspect, the activity of a protein encoded by a gene or member of a gene family may be assayed. Such assays may be conducted by themselves or in conjunction with determining an expression level. In some aspects, it may be desirable to determine an expression level of one or more genes or members of a gene family in Table 1 or 2, while at the same time determining an activity level of one or more proteins encoded by a gene or member of a gene family of Table 1 or 2. The genes or member of gene families for which expression levels are determined may be the same or different as the genes encoding the proteins assayed. Thus, in some embodiments, it may be desirable to determine the expression level of a gene and the activity level of the protein encoded by the gene. In other embodiments, it may be desirable to determine the expression level of one gene while determining the activity level of a protein encoded by another gene. Those skilled in the art will appreciate that the expression and/or activity level of any number of genes and proteins may be determined according to the present invention. In a related aspect, the present invention includes methods of validating a gene or gene family found above, i.e. confirming the gene's or protein's role in e.g. osteoporosis.

In a related aspect, the present invention includes methods of screening for an agent that modulates the differentiation of a MC3T3-E1 cell line, in particular MC3T3-1b clone, into an osteoblast, comprising: preparing a first gene or gene family expression profile and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2 in a cell population comprising one or more MC3T3-E1 cells, in particular MC3T3-1b cells; contacting the cell population with an agent; preparing a second gene or gene family expression profile and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Tables 1 or 2 of the cell population after being contacted with the agent; and comparing the first and second expression profiles and/or activities.

In one aspect, the present invention provides a method of diagnosing a condition characterized by abnormal deposition of bone tissue, comprising detecting the level of expression in a tissue sample of one or more genes or gene families from Table 1 or 2 and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2, wherein differential expression and/or activity is indicative of inadequate bone tissue deposition. The invention includes methods of diagnosing a condition characterized by an abnormal rate of formation of osteoblasts in a patient comprising detecting the level of expression in a tissue sample of one or more genes or gene families from Table 1 or 2 and/or assaying for an activity of a protein encoded by a gene or member of a gene family of

Table 1 or 2; wherein differential expression and/or activity is indicative of an abnormal rate of formation of osteoblasts.

The invention further includes a method of diagnosing osteoporosis in a patient comprising detecting the level of expression in a tissue sample of one or more genes or gene families from Table 1 or 2 and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2; wherein differential expression and/or activity is indicative of osteoporosis.

In another aspect, the present invention also includes methods of monitoring the treatment of a patient with a condition characterized by abnormal bone tissue deposition, comprising administering a pharmaceutical composition to the patient, preparing a gene or gene family expression profile and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2 from a cell or tissue sample from the patient and comparing the patient expression profile and/or activity to an expression profile and/or activity from a MC3T3-E1, in particular MC3T3-1b, cell population or an osteoblast cell population.

In a related aspect, the present invention provides a method of monitoring the treatment of a patient with osteoporosis, comprising administering a pharmaceutical composition to the patient, preparing a gene or gene family expression profile and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2 from a cell or tissue sample from the patient and comparing the patient expression profile and/or activity to an expression profile and/or activity from a MC3T3-E1, in particular MC3T3-1b, cell population or an osteoblast cell population.

In one aspect, the present invention is a method of monitoring the progression of bone tissue deposition in a patient, comprising detecting the level of expression in a tissue sample of one or more genes or gene families from Table 1 or 2 and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2; wherein differential expression and/or activity is indicative of bone tissue deposition.

The invention includes a method of monitoring the treatment of a patient with a condition characterized by abnormal rate of formation of osteoblasts, comprising administering a pharmaceutical composition to the patient, preparing a gene or gene family expression profile and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2 from a cell or tissue sample from the patient and comparing the patient expression profile and/or activity to an expression profile and/or activity from a MC3T3-E1, in particular MC3T3-1b, cell population or an osteoblast cell population.

In a related aspect, the present invention provides a method of treating a patient with a condition characterized by an abnormal rate of formation of osteoblasts, comprising administering to the patient a pharmaceutical composition, wherein the composition alters the expression of at least one gene or gene family in Table 1 or 2 and/or alters an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2, preparing an expression profile and/or assaying for an activity from a MC3T3-E1, in particular MC3T3-1b, cell and comparing the patient expression profile and/or activity to an expression profile and/or activity from an untreated cell population comprising MC3T3-E1, in particular MC3T3-1b, cells.

In another aspect, the present invention also includes methods of treating a patient with a condition characterized by abnormal bone tissue deposition, comprising administering a pharmaceutical composition to the patient; preparing a gene or gene family expression profile and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2 from a cell or tissue sample from the patient comprising MC3T3-E1 cells, in particular MC3T3-1b cells; and comparing the patient expression profile and/or activity to an expression profile and/or activity from an untreated cell population comprising MC3T3-E1 cells, in particular MC3T3-1b cells.

In one aspect, the present invention provides a method of treating a patient with osteoporosis, comprising administering to the patient a pharmaceutical composition, wherein the composition alters the expression of at least one gene or gene family in Table 1 or 2 and/or alters an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2, preparing an expression profile and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2 from a MC3T3-E1 cell, in particular MC3T3-1b cell, and comparing the patient expression profile and/or activity to an expression profile and/or activity from an untreated cell population comprising MC3T3-E1, in particular MC3T3-1b, cells.

Also included in the inventions are methods of screening for an agent capable of ameliorating the effects of osteoporosis, comprising exposing a cell to the agent; and detecting the expression level of one or more genes or gene families from Table 1 or 2 and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2.

In a further aspect, the present invention is a method of screening for an agent capable of modulating the deposition of bone tissue, comprising exposing a cell to the agent and detecting the expression level of one or more genes or gene families from Table 1 or 2

and/or assaying for an activity of a protein encoded by a gene or member of a gene family of Table 1 or 2.

All of these methods may include the step of detecting the expression levels of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more genes or members of the gene families in Table 1 or 2. Preferably, expression of all of the genes or members of the gene families or nearly all of the genes or members of the gene families in Table 1 or 2 may be detected. In a related aspect, the methods of the present invention may comprise the step of assaying for an activity of at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more proteins encoded by a gene or member of a gene family of Table 1 or 2. In some preferred embodiments, the methods of the present invention may comprise both determining an expression level of one or more genes of members of a gene family of Table 1 or 2 and assaying an activity of one or more proteins encoded by a gene or member of a gene family of Table 1 or 2. In some embodiments, the expression level of a gene and the activity level of the protein encoded by the same gene may be determined. In other embodiments, the expression level of at least one gene may be determined while the activity level of at least one protein encoded by a different gene may be determined.

In one aspect, the present invention provides a method for identifying an agent that modulates the differentiation of MC3T3-E1, in particular MC3T3-1b, cells into osteoblasts comprising contacting a cell population with the agent and assaying for at least one activity of at least one gene or the activity of at least one member of a gene family identified in Table 1 or 2. In a related aspect, the present invention provides a method of monitoring the treatment of a patient with a condition characterized by abnormal bone deposition comprising administering a pharmaceutical composition to the patient and assaying for at least one activity of at least one gene or one member of a gene family identified in Table 1 or 2. The present invention also includes a method of diagnosing a condition characterized by the abnormal rate of formation of osteoblast comprising detecting the level of activity of at least one gene or one member of a gene family identified in Table 1 or 2. In some preferred aspects, the present invention encompasses a composition comprising at least two oligonucleotides, wherein each of the oligonucleotides comprises a sequence that specifically hybridizes to one or more genes or members of a gene family in Table 1 or 2. In some aspects, the composition may comprise at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 oligonucleotides, wherein each of the oligonucleotides comprises a sequence that specifically hybridizes to one or more genes or members of a gene family in Table 1 or 2. In some embodiments, one or more of the oligonucleotides may be attached to a solid support.

The solid support may be any known to those skilled in the art including, but not limited to, a membrane, a glass support, a filter, a tissue culture dish, a polymeric material and a silicon support.

In a preferred aspect, the present invention provides a solid support to which is attached at least two oligonucleotides, wherein each of the oligonucleotides comprises a sequence that specifically hybridizes to at least one gene or to at least one member of a gene family in Table 1 or 2. In some embodiments, at least one oligonucleotide is attached covalently to the solid support. In some embodiments, at least one oligonucleotide is attached non-covalently to the solid support. Oligonucleotides may be attached to the solid supports of the invention at any density known to those skilled in the art, for example, at about at least 10 different oligonucleotides in discrete locations per square centimeter, at about at least 100 different oligonucleotides in discrete locations per square centimeter, at about at least 1000 different oligonucleotides in discrete locations per square centimeter and/or at about at least 10,000 different oligonucleotides in discrete locations per square centimeter. The selection of an appropriate density for a given application is a routine procedure for those skilled in the art.

The invention also includes computer systems comprising a database containing information identifying the expression level of one or more members of one or more of the gene families in Table 1 or 2 and/or the activity level of one or more proteins encoded by a gene or by a member of a gene family of Table 1 or 2 in a resting MC3T3-E1, in particular MC3T3-1b, cell and/or a MC3T3-E1, in particular MC3T3-1b, cell differentiating into an osteoblast and/or an osteoblast; and a user interface to view the information. The database may further comprise sequence information for one or more of the genes of one or one or more members of one or more of the gene families of Table 1 or 2. The database may comprise information identifying the expression level for one or more genes or one or more members of one or more of the gene families in the set of gene families expressed in a MC3T3-E1, in particular MC3T3-1b, cell that is not differentiating. The database may comprise information identifying the expression level for one or more genes or one or more members of one or more of the gene families in the set of genes or gene families expressed in a MC3T3-E1, in particular MC3T3-1b, cell that is differentiating into a cell type other than an osteoblast. The database may comprise information identifying the expression level for one or more genes or one or more members of one or more of the gene families in the set of genes or gene families expressed in a precursor stem cell that is differentiating into an osteoblast. The database may further contain or be linked to descriptive information from an

external database, which information correlates said genes and/or gene families to records in the external database.

Moreover, the invention includes methods of using the disclosed computer systems to present information identifying the expression level in a tissue or cell of a set of genes and/or gene families comprising at least one of the genes or gene families in Table 1 or 2, comprising comparing the expression level of at least one gene or gene family in Table 1 or 2 in the tissue or cell to the level of expression of the gene in the database.

The invention also includes methods of using the disclosed computer systems to present information identifying the activity level in a tissue or cell of one or more proteins encoded by one or more genes and/or members of a gene family comprising at least one of the genes or gene families in Table 1 or 2, comprising comparing the activity level of at least one protein encoded by one gene or member of a gene family in Table 1 or 2 in the tissue or cell to the level of activity of the protein in the database.

Detailed description

Many biological functions are accomplished by altering the expression of various genes through transcriptional (e.g. through control of initiation, provision of RNA precursors, RNA processing, etc.) and/or translational control. For example, fundamental biological processes such as cell cycle, cell differentiation and cell death, are often characterized by the variations in the expression levels of groups of genes. Changes ill gene expression also are associated with pathogenesis. For example, the lack of sufficient expression of functional tumor suppressor genes and/or the overexpression of oncogene/protooncogenes could lead to tumorgenesis or hyperplastic growth of cells (Marshall, (1991) Cell 64:313-326; Weinberg, (1991) Science 254:1138-1146). Thus, changes in the expression levels of particular genes (e.g., oncogenes or tumor suppressors) serve as signposts for the presence and progression of various diseases. Monitoring changes in gene expression may also provide certain advantages during drug screening development. Often drugs are screened for the ability to interact with a major target without regard to other effects the drugs have on cells. Often such other effects cause toxicity in the whole animal, which prevent the development and use of the potential drug.

The present inventors have examined cell populations comprising MC3T3-E1 cells, in particular MC3T3-1b cells, that have been induced to differentiate into osteoblasts to identify the global changes in gene expression during this differentiation process. These global changes in gene expression, also referred to as expression profiles, provide useful markers

for diagnostic uses as well as markers that can be used to monitor disease states, disease progression, toxicity, drug efficacy and drug metabolism.

The expression profiles have been used to identify individual genes that are differentially expressed under one or more conditions. In addition, the present invention identifies families of genes that are differentially expressed. As used herein, "gene families" includes, but is not limited to, the specific genes identified by accession number herein, as well as related sequences. Related sequences may be, for example, sequences having a high degree of sequence identity with a specifically identified sequence either at the nucleotide level or at the level of amino acids of the encoded polypeptide. A high degree of sequence identity is seen to be at least about 65% sequence identity at the nucleotide level to said genes, preferably about 80 or 85% sequence identity or more preferably about 90 or 95% or more sequence identity to said genes. With regard to amino acid identity of encoded polypeptides, a high degree of identity is seen to be at least about 50% identity, more preferably about 75% identity and most preferably about 85% or more sequence identity. In particular, related sequences include homologous genes from different organisms. For example, if the specifically identified gene is from a non-human mammal, the gene family would encompass homologous genes from other mammals including humans. If the specifically identified gene is a human gene, gene family would encompass the homologous gene from different organisms. Those skilled in the art will appreciate that a homologous gene may be of different length and may comprise regions with differing amounts of sequence identity to a specifically identified sequence.

Assay Formats: The genes and sequences identified as being differentially expressed in the cell population induced to differentiate as well as related sequences may be used in a variety of nucleic acid detection assays to detect or quantitate the expression level of a gene or multiple genes in a given sample. For example, traditional Northern blotting, ribonuclease protection, RT-PCR, QPCR (quantitative RT-PCR), real-time PCR such as TaqMan System and differential display methods may be used for detecting gene expression levels. Those methods are useful for some embodiments of the invention. However, methods and assays of the invention are most efficiently designed with hybridization-based methods for detecting the expression of a large number of genes. Any hybridization assay format may be used, including solution-based and solid support-based assay formats. Solid supports containing oligonucleotide probes for differentially expressed genes of the invention can be filters, polyvinyl chloride dishes, silicon or glass based chips, etc. Such supports and hybridization methods are widely available, for example, those disclosed by WO 95/11755. Any solid

surface to which oligonucleotides can be bound, either directly or indirectly, either covalently or non covalently, can be used. A preferred solid support is a high density array or DNA chip. These contain a particular oligonucleotide probe in a predetermined location on the array. Each predetermined location may contain more than one molecule of the probe, but each molecule within the predetermined location has an identical sequence. Such predetermined locations are tended features. There may be, for example, from 2, 10, 100, 1000 to 10000, 100000 or 400000 of such features on a single solid support. The solid support, or the area within which the probes are attached may be any convenient size and may preferably be on the order of a square centimeter. Oligonucleotide probe arrays for expression monitoring can be made and used according to any techniques known in the art (see for example, Lockhart et al., (1996)

Nat. Biotech. 14, 1675-1680, McGall et al., (1996) Proc. Nat. Acad. Sci. USA 93, 13555-13460). Such probe arrays may contain at least two or more oligonucleotides that are complementary to or hybridize to two or more of the genes described in Table 1 or 2. For instance, such arrays may also contain oligonucleotides that are complementary or hybridize to at least about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 50, 70 or more the genes described herein. The genes which are assayed according to the present invention are typically in the form of mRNA or reverse transcribed mRNA. The genes may be cloned or not. The genes may be amplified or not. The cloning itself does not appear to bias the representation of genes within a population. However, it may be preferable to use polyadenylated RNA as a source, as it can be used with less processing steps.

Table 1 and 2 provides the Accession numbers and name for the sequences of the differentially expressed markers. The sequences of the genes in GenBank are expressly incorporated herein.

Probes based on the sequences of the genes described above may be prepared by any commonly available method. Oligonucleotide probes for interrogating the tissue or cell sample are preferably of sufficient length to specifically hybridize only to appropriate, complementary genes or transcripts. Typically the oligonucleotide probes will be at least 10, 12, 14, 16, 18, 20 or 25 nucleotides in length. In some cases longer 20 probes of at least 30, 40 or 50 nucleotides will be desirable.

As used herein, oligonucleotide sequences that are complementary to one or more of the genes and/or gene families described in Table 1 or 2, refer to oligonucleotides that are capable of hybridizing under stringent conditions to at least part of the nucleotide sequences of said genes. Such hybridizable oligonucleotides will typically exhibit at 25 least about 75%

sequence identity at the nucleotide level to said genes, preferably about 80 or 85% sequence identity or more preferably about 90 or 95% or more sequence identity to said genes.

"Bind(s) substantially" refers to complementary hybridization between a probe nucleic acid and a target nucleic acid and embraces minor mismatches that can be accommodated by reducing the stringency of the hybridization media to achieve the desired detection of the target polynucleotide sequence.

The terms "background" or "background signal intensity" refer to hybridization signals resulting from non-specific binding, or other interactions, between the labeled target nucleic acids and components of the oligonucleotide array (e.g. the oligonucleotide probes, control probes, the array substrate, etc.). Background signals may also be produced by intrinsic fluorescence of the array components themselves. A single background signal can be calculated for the entire array, or a different background signal may be calculated for each target nucleic acid. In a preferred embodiment, background is calculated as the average hybridization signal intensity for the lowest 5 to 10% of the probes in the array, or, where a different background signal is calculated for each target gene, for the lowest 5 to 10% of the probes for each gene. Of course, one of skill in the art will appreciate that where the probes to a particular gene hybridize well and thus appear to be specifically binding to a target sequence, they should not be used in a background signal calculation. Alternatively, background may be calculated as the average hybridization signal intensity produced by hybridization to probes that are not complementary to any sequence found in the sample (e.g., probes directed to nucleic acids of the opposite sense or to genes not found in the sample such as bacterial genes where the sample is mammalian nucleic acids). Background can also be calculated as the average signal intensity produced by regions of the array that lack any probes at all.

The phrase "hybridizing specifically to" refers to the binding, duplexing, or hybridizing of a molecule substantially to or only to a particular nucleotide sequence or sequences under stringent conditions when that sequence is present in a complex mixture (e.g. total cellular) DNA or RNA.

Assays and methods of the invention may utilize available formats to simultaneously screen at least about 100, preferably about 1000, more preferably about 25 10,000 and most preferably about 1,000,000 different nucleic acid hybridizations.

The terms "mismatch control" or "mismatch probe" refer to a probe whose sequence is deliberately selected not to be perfectly complementary to a particular target sequence. For

each mismatch (MM) control in a high- density array there typically exists a corresponding perfect match (PM) probe that is perfectly complementary to the same particular target sequence. The mismatch may comprise one or more bases.

While the mismatch(s) may be located anywhere in the mismatch probe, terminal mismatches are less desirable as a terminal mismatch is less likely to prevent hybridization of the target sequence. In a particularly preferred embodiment, the mismatch is located at or near the center of the probe such that the mismatch is most likely to destabilize the duplex with the target sequence under the test hybridization conditions.

The term "perfect match probe" refers to a probe that has a sequence that is perfectly complementary to a particular target sequence. The test probe is typically perfectly complementary to a portion (subsequence) of the target sequence. The perfect match (PM) probe can be a "test probe" or a "normalization control" probe, an expression level control probe and the like. A perfect match control or perfect match probe is, however, distinguished from a "mismatch control" or "mismatch probe" as defined herein.

As used herein a "probe" is defined as a nucleic acid, capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, usually through complementary base pairing, usually through hydrogen bond formation. As used herein, a probe may include natural (i.e., A, G. U. C or T) or modified bases (7-deazaguanosine, inosine, etc.). In addition, the bases in probes may be joined by a linkage other than a phosphodiester bond, so long as it does not interfere with hybridization. Thus, probes may be peptide nucleic acids in which the constituent bases are joined by peptide bonds rather than phosphodiester linkages.

The term "stringent conditions" refers to conditions under which a probe will hybridize to its target subsequence, but with only insubstantial hybridization to other sequences or to other sequences such that the difference may be identified. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. Typically, stringent conditions will be those in which the salt concentration is at least about 0.01 to 1.0 M sodium ion concentration (or other salts) at pH 7.0 to 8.3 and 30 the temperature is at least about 30°C for short probes (e.g. 10 to 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide.

The 'percentage of sequence identity" or "sequence identity" is determined by comparing two optimally aligned sequences or subsequences over a comparison window or span, wherein the portion of the polynucleotide sequence in the comparison window 5 may optionally comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical residue (e.g., nucleic acid base or amino acid residue) occurs in both sequences to yield the number of matched positions, dividing the number of matched 10 positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity. Percentage sequence identity can be calculated by the local homology algorithm of Smith & Waterman, (1981) Adv. Appl. Math. 2:482-485; by the homology alignment algorithm of Needleman & Wunsch, (1970) J. Mol. Biol. 48:443-445; or by computerized implementations of these algorithms (GAP & BESTFIT in the GCG Wisconsin Software Package, Genetics Computer Group) or by manual alignment and visual inspection. Percentage sequence identity when calculated using the programs GAP or BESTFIT is calculated using default gap weights. The BESTFIT program has two alignment variables, the gap creation penalty and the gap extension penalty, which can be modified to alter the stringency of a nucleotide and/or amino acid alignment produced by the program. Parameter values used in the percent identity determination were default values previously established for version 8.0 of BESTFIT (see Dayhoff, (1979) Atlas of Protein Sequence and Structure, National Biomedical Research Foundation, pp. 353 25 358).

Probe design: One of skill in the art will appreciate that an enormous number of array designs are suitable for the practice of this invention. In some preferred embodiments, a high density array may be used. The high density array will typically include a number of probes that specifically hybridize to the sequences of interest (see WO 99/32660 for methods of producing probes for a given gene or genes). In addition, in a preferred embodiment, the array will include one or more control probes. High density array chips of the invention include "test probes" as defined herein. Test probes could be oligonucleotides that range from about 5 to about 45 or 5 to about 5 500 nucleotides, more preferably from about 10 to about 40 nucleotides and most preferably from about 15 to about 40 nucleotides in length. In other particularly preferred embodiments, the probes are 20 or 25 nucleotides in length. In another preferred embodiment, test probes are double or single strand nucleic acid sequences, preferably DNA sequences. Nucleic acid sequences may be isolated or cloned

from natural sources or amplified from natural sources using native nucleic acid as templates. These probes have sequences complementary to particular subsequences of the genes whose expression they are designed to detect. Thus, the test probes are capable of specifically hybridizing to the target nucleic acid they are to detect. In addition to test probes that bind the target nucleic acid(s) of interest, the high density array can contain a number of control probes. The control probes fall into three categories referred to herein as (1) normalization controls; (2) expression level controls; and (3) mismatch controls. Normalization controls are oligonucleotide or other nucleic acid probes that are complementary to labeled reference oligonucleotides or other nucleic acid sequences that are added to the nucleic acid sample to be screened. The signals obtained from the normalization controls after hybridization provide a control for variations in hybridization conditions, label intensity, "reading" efficiency and other factors that may cause the signal of a perfect hybridization to vary between arrays. In a preferred embodiment, signals (e.g., fluorescence intensity) read from all other probes in the array are divided by the signal (e.g., fluorescence intensity) from the control probes thereby normalizing the measurements. Virtually any probe may serve as a normalization control. However, it is recognized that hybridization efficiency varies with base composition and probe length. Preferred normalization probes are selected to reflect the average length of the other probes present in the array, however, they can be selected to cover a range of lengths. The normalization control(s) can also be selected to reflect the (average) base composition of the other probes in the array, however in a preferred embodiment, only one or a few probes are used and they are selected such that they hybridize well (i. e., no secondary structure) and do not match any target-specific probes. Expression level controls are probes that hybridize specifically with constitutively expressed genes in the biological sample. Virtually any constitutively expressed gene provides a suitable target for expression level controls. Typically expression level control probes have sequences complementary to subsequences of constitutively expressed "housekeeping genes" including, but not limited to the actin gene, the transferrin receptor gene, the GAPDH gene, and the like. Mismatch controls may also be provided for the probes to the target genes, for expression level controls or for normalization controls. Mismatch controls are oligonucleotide probes or other nucleic acid probes identical to their corresponding test or control probes except for the presence of one or more mismatched bases. A mismatched base is a base selected so that it is not complementary to the corresponding base in the target sequence to which the probe would otherwise specifically hybridize. One or more mismatches are selected such that under appropriate

hybridization conditions (e.g., stringent conditions) the test or control probe would be expected to hybridize with its target sequence, but the mismatch probe would not hybridize (or would hybridize to a significantly lesser extent). Preferred mismatch probes contain a central mismatch. Thus, for example, where a probe is a twenty-mar, a corresponding mismatch probe will have the identical sequence except for a single base mismatch (e.g., substituting a G. C or T for an A) at any of positions six through fourteen (the central mismatch). Mismatch probes thus provide a control for non-specific binding or cross hybridization to a nucleic acid in the sample other than the target to which the probe is directed. Mismatch probes also indicate whether a hybridization is specific or not. For example, if the target is present the perfect match probes should be consistently brighter than the mismatch probes. In addition, if all central mismatches are present, the mismatch probes can be used to detect a mutation. The difference in intensity between the perfect match and the mismatch probe provides a good measure of the concentration of the hybridized material.

Nucleic Acid Samples: As is apparent to one of ordinary skill in the art, nucleic acid samples, which may be DNA and/or RNA, used in the methods and assays of the invention may be prepared by any available method or process. Methods of isolating total mRNA are well letdown to those of skill in the art. For example, methods of isolation and purification of nucleic acids are described in detail in Chapter 3 of Tijssen, (1993) Laboratory Techniques in Biochemistry and Molecular Biology: Hybridization With Nucleic Acid Probes, Elsevier Press. Such samples include RNA samples, but also include cDNA synthesized from a mRNA sample isolated from a cell or tissue of interest. Such samples also include DNA amplified from the cDNA, and RNA transcribed from the amplified DNA. One of skill in the art would appreciate that it is desirable to inhibit or destroy RNase present in homogenates before homogenates can be used. Biological samples may be of any biological tissue or fluid or cells from any organism as well as cells raised in. vitro, such as cell lines and tissue culture cells. Frequently, the sample will be a "clinical sample" which is a sample derived from a patient. Typical clinical samples include, but are not limited to, sputum, blood, blood cells (e.g., white cells, serum markers), tissue or fine needle biopsy samples, urine, peritoneal fluid, and pleural fluid, or cells therefrom. Bone changes can be also observed with non-invasive scanning techniques. Biological samples may also include sections of tissues, such as frozen sections or formalin fixed sections taken for histological purposes.

Forming High Density Arrays: Methods of forming high density arrays of oligonucleotides with a minimal 25 number of synthetic steps are known. The oligonucleotide

analogue array can be synthesized on a solid substrate by a variety of methods, including, but not limited to, light- directed chemical coupling, and mechanically directed coupling (see U.S. Patent 5,143,854). in brief, the light-directed combinatorial synthesis of oligonucleotide arrays on a 30 glass surface proceeds using automated phosphoramidite chemistry and chip masking techniques. In one specific implementation, a glass surface is derivatized with a silane reagent containing a functional group, e.g. a hydroxyl or amine group blocked by a photolabile protecting group. Photolysis through a photolithogaphic mask is used selectively to expose functional groups which are then ready to react with incoming 5' photoprotected nucleoside phosphoramidites. The phosphoramidites react only with those sites which are illuminated (and thus exposed by removal of the photolabile blocking group). Thus, the phosphoramidites only add to those areas selectively exposed from the preceding step. These steps are repeated until the desired array of sequences have been synthesized on the solid surface. Combinatorial synthesis of different oligonucleotide analogues at different locations on the array is determined by the pattern of illumination during synthesis and the order of addition of coupling reagents. In addition to the foregoing, additional methods which can be used to generate an array of oligonucleotides on a single substrate are described in WO 93/09668. High density nucleic acid arrays can also be fabricated by depositing premade or natural nucleic acids in predetermined positions. Synthesized or natural nucleic acids are deposited on specific locations of a substrate by light directed targeting and oligonucleotide directed targeting. Another embodiment uses a dispenser that moves from region to region to deposit nucleic acids in specific spots.

Hvbridization: Nucleic acid hybridization simply involves contacting a probe and target nucleic acid under conditions where the probe and its complementary target can form stable hybrid duplexes through complementary base pairing (see WO 99/32660). The nucleic acids that do not form hybrid duplexes are then washed away leaving the hybridized nucleic acids to be detected, typically through detection of an attached detectable label. It is generally recognized that nucleic acids are denatured by increasing the temperature or decreasing the salt concentration of the buffer containing the nucleic acids. Under low stringency conditions (e.g. , low temperature and/or high salt) hybrid duplexes (e.g., DNA:DNA, RNA:RNA or RNA:DNA) will form even where the annealed sequences are not perfectly complementary. Thus specificity of hybridization is reduced at lower stringency. Conversely, at higher stringency (e.g., higher temperature and/or lower salt and/or in the presence of destabilizing reagents) successful hybridization tolerates fewer mismatches. One of skill in the art will appreciate that hybridization conditions may be selected to provide any degree of stringency.

In a preferred embodiment, hybridization is performed at low stringency in this case in 6x SSPE-T at 37°C (0.005% Triton x-100) to ensure hybridization and then subsequent washes are performed at higher stringency 5 (e.g., 1 x SSPE-T at 37°C) to eliminate mismatched hybrid duplexes. Successive washes may be performed at increasingly higher stringency (e.g., down to as low as 0.25x SSPET at 37°C to 50°C) until a desired level of hybridization specificity is obtained. Stringency can also be increased by addition of destabilizing agents such as formamide. Hybridization specificity may be evaluated by comparison of hybridization to the test probes with hybridization to the various controls that can be present (e.g., expression level control, normalization control, mismatch controls, etc.). In general, there is a trade-off between hybridization specificity (stringency) and signal intensity. Thus, in a preferred embodiment, the wash is performed at the highest stringency that produces consistent results and that provides a signal intensity greater than approximately 10% of the background intensity. Thus, in a preferred embodiment, the hybridized array may be washed at successively higher stringency solutions and read between each wash. Analysis of the data sets thus produced will reveal a wash stringency above which the hybridization pattern is not appreciably altered and which provides adequate signal for the particular oligonucleotide probes of interest.

Signal Detection: The hybridized nucleic acids are typically detected by detecting one or more labels attached to the sample nucleic acids. The labels may be incorporated by any of a number of means well known to those of skill in the art (see WO 99/32660).

Databases: The present invention includes relational databases containing sequence information, for instance, for the genes and members of the gene families of Table 1 or 2 as well as gene expression information in various tissue samples saved on computer readable medium and/or a user interface. Databases may also contain information associated with a given sequence or tissue sample such as descriptive information about the gene associated with the sequence information, or descriptive information concerning the clinical status of the tissue sample, or the patient from which the sample was derived. The database may be designed to include different parts, for instance a sequence database and a gene expression database. Methods for the configuration and construction 5 of such databases are widely available, for instance, see U.S. Patent 5,953,727, which is herein incorporated by reference in its entirety. The databases of the invention may be linked to an outside or external database. In a preferred embodiment, the external database is GenBank and the associated databases maintained by the National Center for Biotechnology Information (NCBI). Any appropriate computer platform may be used to perform the necessary comparisons between

sequence information, gene expression information and any other information in the database or provided as an input. For example, a large number of computer workstations are available from a variety of manufacturers, such has those available from Silicon Graphics. Client/server environments, database servers and networks are also widely available and appropriate platforms for the databases of the invention. The databases of the invention may be used to produce, among other things, electronic Northerns that allow the user to determine the cell type or tissue in which a given gene is expressed and to allow determination of the abundance or expression level of a given gene in a particular tissue or cell. The databases of the invention may also be used to present information identifying the expression level in a sample of a set of genes comprising one or more of the sequences of genes or members of the gene families of Table 1 or 2, comprising comparing the expression level of at least one gene or member of a gene family of Table 1 or 2 in the sample to the level of expression of the gene in the database. Such methods may also be used in the drug or agent screening assays as described below.

Diagnostic Uses for the Differentiation Markers: As described above, the genes and gene expression information provided in Table 1 or 2 may be used as diagnostic markers for the prediction or identification of the differentiation state of a sample comprising precursor stem cells. For instance, a tissue sample may be assayed by any of the methods described above, and the expression levels from a gene or member of a gene family from Table 1 or 2 may be compared to the expression levels found in un- differentiated precursor stem cells and/or precursor stem cells induced to differentiate into osteoblasts and/or precursor stem cells induced to differentiate into a cell type other than an osteoblast and/or osteoblasts. The comparison of expression data, as well as available sequence or other information may be done by researcher or diagnostician or may be done with the aid of a computer and databases as described above. Such methods may be used to diagnose or identify conditions 15 characterized by abnormal bone deposition, re-absorption and/or abnormal rates of osteoblast differentiation. Those skilled in the art will appreciate that a wide variety of conditions are associated with abnormal bone deposition or loss. Such conditions include, but are not limited to, osteoporosis, osteopenia, osteodystrophy, and various other osteopathic conditions. The methods of the present invention will be particularly useful in diagnosing or monitoring the treatment of bone diseases, including, but not limited to post-menopausal osteoporosis (PMO), glucocorticoid-induced osteoporosis (GIO), male osteoporosis, acute bone loss, tumor-induced osteolysis, Paget's disease, and osteogenesis imperfecta.

Agents which modulate the expression of one or more the genes or gene families identified in Table 1 or 2 and/or modulate the activity of one or more of the proteins encoded by one or more of the genes or members of a gene family identified in Table 1 or 2 will be useful in treatment of the conditions. Some preferred embodiments, the present invention may be used to diagnose and/or monitor the treatment of drug-induced abnormalities in bone formation or loss. For example, at present a combination of cyclosporine with prednisone is given to patients who have received an organ transplant in order to suppress tissue rejection. The combination causes rapid bone loss in a manner different than that observed with prednisone alone (such as elevated level of serum osteocalcin and 1,25(OH)2-Vitamin D in patients treated with cyclosporine but not in patients treated with prednisone). Other drugs are also known to effect bone formation or loss. The anticonvulsant drugs diphenylhydantoin, phenobarbital and carbamazepine, and combination of these drugs, cause alterations in calcium metabolism. A decrease in bone density is observed in patients taking anticonvulsant drugs. Although heparin is an effective therapy for thromboembolic disorders, increased incidences of osteoporotic fractures have been reported in patients with heparin therapy hence the present invention will be useful to monitor patients undergoing heparin treatment. Other embodiments of the present invention allow the diagnosis and/or monitoring of the treatment of other conditions that involve altered bone metabolism. For example, idiopathic juvenile osteoporosis (IJO) is a generalized decrease in mineralized bone in the absence of rickets or excessive bone resorption and typically occurs in children before the onset of puberty. In addition, thyroid diseases have been linked bone loss. A decrease in bone mass has been shown in patients with thyrotoxicosis causing these individuals to be at increased risk of having fractures. These individuals also sustain fractures at an earlier age than individuals who have never been thyrotoxic. Other conditions in which the present invention will be useful include multiple myeloma and leukemia. Nearly 60% of patients with multiple myelomas have bone fractures with focal and lytic bone lesions and osteosclereotic bone lesions. Leukemia may also be associated with diffuse osteopenia and vertebral fracture in patients with acute lymphoblastic leukemia. Another situation in which the present invention will be useful is the diagnosis and/or monitoring of the treatment of skeletal disease linked to breast cancer. Breast cancer frequently metastasizes to the skeleton and about 70% of patients with advanced cancer develop symptomatic skeletal disease. Moreover, the anticancer treatments presently in use have been shown to lead to early menopause and bone loss when given to premenopausal women. The present invention will be useful in diagnosing and/or monitoring the treatment of

chronic anemia associated with abnormal bone formation or loss. Homozygous beta-thalassemia is usually described as an example of chronic anemia predisposing to osteoporosis. Patients with thalassemia have expansion of bone marrow space with thinning of the adjacent trabeculae. The present invention will be useful in diagnosing and/or monitoring the treatment of mastocytosis. Skeletal symptoms (osteopenia and vertebral fracture) are present in 60 to 75% of the patients with systemic mast cell disease. Other conditions in which the present invention will find application are: Fanconi syndrome where osteomalacia is a common feature; fibrous dysplasia, McCune-Albright syndrome refers to patients with fibrous dysplasia with a sporadic, developmental disorder characterized by a unifocal or multifocal expanding fibrous lesion of bone forming mesenchyme that often results in pain, fracture or deformity, osteogenesis imperfecta (OI, also called brittle bone disease) is associated with recurrent fractures and skeletal deformity, various skeletal dysplasias, i.e., osteochondroplasia which is characterized by abnormal development of cartilage and/or bone and other diseases such as gingival diseases such as gingivitis and periodontitis, Paget's disease, hypercalcemia of malignancy, e.g. tumour-induced hypercalcemia and metabolic bone disease, achodroplasia, mucopolysacchaidoses, dysostosis and ischemic bone diseases. The present invention will be particularly useful by providing one or markers which may be used as markers of bone turnover to determine osteoporosis. The present invention may also be used in in vitro assays or treatments as a marker of osteoblast differentiation and/or proliferation.

The agents of the present invention may be used for a variety of purposes. In a preferred embodiment, they may be used in fracture repair of all types, i.e., non-union fractures, spinal fusion, accelerated healing of all types fractures from minor greenstick or compression fractures to comminuted, complicated fractures. Both local administrations to these fractures as well as parenteral administration which increases cartilage and bone formation, increases bone mass, and increases bone strength rapidly may be used. Another preferred embodiment of the present invention is the use of bone formation modulating agents in periodontal disease and/or for increasing bone around teeth.

Use of the Differentiation Markers for Monitoring Disease Progression: As described above, the genes and gene expression information provided in Table 1 or 2 may also be used as markers for the monitoring of disease progression, such as osteoporosis. For instance, a tissue sample may be assayed by any of the methods described above, and the expression levels for a gene or member of a gene family from Table 1 or 2 may be compared to the expression levels found in undifferentiated precursor stem cells and/or

precursor stem cells induced to differentiate into osteoblasts and/or precursor stem cells induced to differentiate into a cell type other than an osteoblast and/or osteoblasts. The comparison of the expression data, as well as available sequence or other information may be done by researcher or diagnostician or may be done with the aid of a computer and databases as described above. The markers of the invention may also be used to Pack or predict the progress or efficacy of a treatment regime in a patient. For instance, a patient's progress or response to a give drug may be monitored by creating a gene expression profile from a tissue or cell sample after treatment or administration of the drug. The gene expression profile may then be compared to a gene expression profile prepared from un-differentiated precursor stem cells and/or precursor stem cells induced to differentiate into osteoblasts and/or precursor stem cells induced to differentiate into a cell type other than an osteoblast and/or osteoblasts and/or from tissue or cells from the same patient before treatment. The gene expression profile may be made from at least one gene, preferably more than one gene, and most preferably all or nearly all of the genes in Table 1 or 2.

Use of the Differentiation Markers for Drug Screening: According to the present invention, the genes identified in Table 1 or 2 may be used as markers to evaluate the effects of a candidate drug or agent on a cell. A candidate drug or agent can be screened for the ability to stimulate the transcription or expression of a given marker or markers or to down- regulate or counteract the transcription or expression of a marker or markers. For instance, agents that modulate, induce or inhibit gene expression in a sample to that which resembles a gene expression profile in an osteoblast differentiated cell population may be screened for the ability to modulate the differentiation process, bone depositions, etc. According to the present invention one can also compare the specificity of a drug effect by looking at the number of markers which the drug has and comparing them. More specific drugs will have less transcriptional targets. Similar sets of markers identified for two drugs indicates a similarity of effects. Assays to monitor the expression of a marker or markers as defined in Table 1 or 2 may utilize any available means of monitoring for changes in the expression level of the nucleic acids of the invention. As used herein, an agent is said to modulate the expression of a nucleic acid of the invention if it is capable of up- or down- regulating expression of the nucleic acid in a cell. In one assay format, gene chips containing probes to one or more genes or members of a gene family from Table 1 or 2 may be used to directly monitor or detect changes in gene expression in the treated or exposed cell as described in more detail above. In another format, cell lines that contain reporter gene fusions between the open reading frame and/or 5' - 3' regulatory regions of a gene or

member of a gene family in Table 1 or 2 and any assayable fusion partner may be prepared. Numerous assayable fusion partners are known and readily available including the firefly luciferase gene and the gene encoding chloramphenicol acetyltransferase (Slam et al., (1990) Anal. Biochem. 15 188:245-254). Cell lines containing the reporter gene fusions are then exposed to the agent to be tested under appropriate conditions and time. Differential expression of the reporter gene between samples exposed to the agent and control samples identifies agents which modulate the expression of the nucleic acid. Additional assay formats may be used to monitor the ability of the agent to modulate the expression of a gene or member of a gene family identified in Table 1 or 2. For instance, as described above, mRNA expression may be monitored directly by hybridization of probes to the nucleic acids of the invention. Cell lines are exposed to the agent to be tested under appropriate conditions and time and total RNA or mRNA is isolated by standard procedures such those disclosed in Sambrook et al., (1989) 25 Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press. In another assay format, cells or cell lines are first identified which express the gene products of the invention physiologically. Cells and/or cell lines so identified would be expected to comprise the necessary cellular machinery such that the fidelity of modulation of the transcriptional apparatus is maintained with regard to exogenous contact of agent with appropriate surface transduction mechanisms and/or the cytosolic cascades. Such cell lines may be, but are not required to be, bone marrow derived. Further, such cells or cell lines may be transduced or transfected with an expression vehicle (e.g., a plasmid or viral vector) construct comprising an operable non-translated 5'-promoter containing end of the structural gene encoding the instant gene products fused to one or more antigenic fragments, which are peculiar to the instant gene products, wherein said fragments are under the transcriptional control of said promoter and are expressed as polypeptides whose molecular weight can be distinguished from the naturally occurring polypeptides or may further comprise an immunologically distinct tag or some other detectable marker or tag. Such a process is well known in the art (see Sambrook et al., (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press). Cells or cell lines transduced or transfected as outlined above are then contacted with agents under appropriate conditions; for example, the agent comprises a pharmaceutically acceptable excipient and is contacted with cells comprised in an aqueous physiological buffer such as phosphate buffered saline (PBS) at physiological pH, Eagles balanced salt solution (BSS) at physiological pH, PBS or BSS comprising serum or conditioned media comprising PBS or BSS and/or serum incubated at 37°C. Said conditions may be modulated as deemed necessary by one of skill

in the art. Subsequent to contacting the cells with the agent, said cells are disrupted and the polypeptides of the lysate are fractionated such that a polypeptide fraction is pooled and contacted with an antibody to be further processed by immunological assay (e.g., ELISA, immunoprecipitation or Western blot). The pool of proteins isolated from the "agent contacted" sample is then compared with a control sample Where only the excipient is contacted with the cells and an increase or decrease in the immunologically generated signal from the "agent- contacted" sample compared to the control is used to distinguish the effectiveness of the agent. Another embodiment of the present invention provides methods for identifying agents that modulate the levels or at least one activity of a protein(s) encoded by the genes in Table 1 or 2. Such methods or assays may utilize any means of monitoring or detecting the desired activity. In one format, the relative amounts of a protein of the invention between a cell population that has been exposed to the agent to be tested compared to an un-exposed control cell population may be assayed. In this format, probes such as specific antibodies are used to monitor the differential expression of the protein in the different cell populations. Cell lines or populations are exposed to the agent to be tested under appropriate conditions and time. Cellular lysates may be prepared from the exposed cell 5 line or population and a control, unexposed cell line or population. The cellular lysates are then analyzed with the probe, such as a specific antibody. Agents that are assayed in the above methods can be randomly selected or rationally selected or designed. As used herein, an agent is said to be randomly selected when the agent is chosen randomly without considering the specific sequences involved in the association of the a protein of the invention alone or with its associated substrates, binding partners, etc. An example of randomly selected agents is the use a chemical library or a peptide combinatorial library, or a growth broth of an organism. As used herein, an agent is said to be rationally selected or designed when the agent is chosen on a nonrandom basis which takes into account the sequence of the target site and/or its conformation in connection with the agent's action. Agents can be rationally selected or rationally designed by utilizing the peptide sequences that make up these sites. For example, a rationally selected peptide agent can be a peptide whose amino acid sequence is identical to or a derivative of any functional consensus site. The agents of the present invention can be, as examples, peptides, small molecules, vitamin derivatives, as well as carbohydrates. Dominant negative proteins, DNA encoding these proteins, antibodies to these proteins, peptide fragments of these proteins or mimics of these proteins may be introduced into cells to affect function.

"Mimic" used herein refers to the modification of a region or several regions of a peptide molecule to provide a structure chemically different from the parent peptide but topographically and functionally similar to the parent peptide (see Meyers, (1995) Molecular Biology and Biotechnology, VCH Publishers, 659-664). A skilled artisan can readily recognize that there is no limit as to the structural nature of the agents of the present invention. Without further description, it is believed that one of ordinary skill in the art can, using the preceding description and the following illustrative examples, make and utilize the compounds of the present invention and practice the claimed methods. The following working examples therefore, specifically point out the preferred embodiments of the present invention, and are not to be construed as limiting in any way the remainder of the disclosure.

Examples

Methods:

Cell culture and stimulation: MC3T3-E1 cell line was a kind gift of T. Kokkubo (Novartis, Japan) (Sudo et al., 1983, J Cell Biol; 96:191-198). The MC3T3-1b clone was generated by stable transfection of Cbfa1 DNA response element OSE2 upstream of the reporter gene Luciferase into MC3T3-E1 cell line mentioned above. The MC3T3-1b cell clone was selected on the basis of activation of the Luciferase gene by the osteogenic stimulus, comprising 200 ng/ml BMP-2 (bone morphogenetic protein - 2), 50 µM ascorbic acid and 10 mM β-glycerophosphate. The cells were grown in α-MEM with 10% fetal calf serum (FCS, Gibco), 1% penicilin/streptomycin (Pen/Strep) and 1% L-Glutamine (L-Glu) in T175 flasks (40 ml/flask). The stimulation was done in the same medium.

For RNA isolation, cells were plated on 6 cm dishes for ($3\times10^{5\ cells}$/dish in 5 ml medium), for alkaline phosphatase staining on 48-well plates (1×10^4 cells/well in 1 ml medium) and for Alizarin red S staining on 12-well plates (5×10^4 cells/well in 3 ml medium). The cells were grown to confluence for 3 days at 37°C / 5% CO_2, and then stimulated with 10 mM β-glycerophosphate (GP, Sigma), 50 µM ascorbic acid (AA, Wako) and 200 ng/ml BMP-2 (Nico Cerletti, Novartis). The control cells were stimulated with 10 mM β-glycerophosphate. For Alizarin Red S staining, the medium together with stimulators was changed twice weekly.

RNA isolation: RNA was extracted from non-stimulated confluent cells (day 0), and from the cells treated with GP/AA/BMP-2 or GP alone for 1 and 3 days. The cells were harvested in the Lysis Buffer containing guanidinium-isothiocyanate. The total RNA was extracted, treated with DNAse I and purified according to manufacturer's protocol (RNeasy mini kit, QIAGEN; Basel, Switzerland).

Alkaline phosphatase staining: Three days after stimulation, the cells were washed twice with PBS, fixed with formalin /methanol / H_2O (1:1:1.5), 0.5 ml/well, incubated 15 min at room temperature, and washed 3 times with water. For staining, one FAST BCIP/NBT tablet from Sigma (5-bromo-4-chloro-3-indolyl phosphate / nitro blue tetrazolium, alkaline phosphatase substrate) was dissolved in 10 ml of water. To the fixed cultures, 0.5 ml of substrate solution was added and incubated for 15 min at room temperature. After staining, the cultures were washed 3 times with water and air-dried.

Alizarin red S staining for mineralization: Fourteen days after stimulation, the cells were washed twice in PBS, fixed with formalin /methanol / H_2O (1:1:1.5), 0.5 ml/well, 15 minutes at room temperature, and 3 times washed with water. Saturated Alizarin Red S solution was

filtered, 1.5 ml was added per well, and incubated for 15 min at room temperature. The cells were washed 4-5 times with water and air-dried.

Quantitative radioactive RT-PCR (qrRT-PCR): qrRT-PCR was used to analyze expression profiles of the marker genes prior to microarray analysis, and to confirm microarray data for some selected genes.First strand complementary DNA was synthesized according to the standard protocol. Briefly, 10 units of RNAse inhibitor (ROCHE Molecular Diagnostics) and 100 µg of random hexanucleotides (Amersham Pharmacia Biotech) were added to 1 µg of DNAse I-treated total cellular RNA. Samples were denatured for 5 min at 65°C and chilled on ice. Then, samples were filled to 20 µl with a nuclease-free solution containing another 10 units of RNAse inhibitor, 2.5 mM of each dNTP, 50 mM Tris-HCl pH 8.3, 60 mM KCl, 10 mM MgCl$_2$ and 1 mM DTT. 20 units of Avian Myeloblastosis Virus reverse transcriptase (Stratagene) was added (cDNA) or not (RT-) to each sample. Reverse transcription reaction was performed for 2 h at 42°C, and stopped by incubation for 5 min at 95°C. Samples were diluted 5-fold with nuclease-free water and stored at -80°C until use. One µl of cDNA or RT-sample was used as a PCR template. PCR reaction was performed in a final volume of 25 µl, containing 100 µM of each dNTP, 1 µCi of α [^{32}P]-dATP, 1 µM of each primer and 1.25 units of "Hot start" thermostable DNA polymerase and corresponding reaction buffer (FastStart Taq, ROCHE Molecular Diagnostics). For PCR analysis of PTHR, Dlx2, RAMP1, JunB, Fra-1, and Wnt6, the reaction mix contained in addition 5 % glycerol. The amplification protocol was the following: initial step of 5 min at 94°C, 12-33 cycles of denaturation at 94° C for 1 min, annealing at 57/60° C (all genes at 57° C, except PTHR, Dlx2, RAMP1, JunB, Fra-1, at 60° C) for 1 min, and extension at 72° C for 1 min 20 s. The amplification was terminated with the final incubation step at 72° C for 10 min. Aliquots of PCR products were mixed with a loading buffer (final concentrations: 5 % glycerol, 10 mM EDTA, 0.01 % SDS, 0.025 % xylene cyanol and bromophenol blue dyes) and analyzed on 8 % polyacrylamide gels. The gels were vacuum-dried, exposed to phosphor-storage screens (Molecular Dynamics) and imaged by PhosphorImager (Molecular Dynamics). The signals on images were quantified by the ImageQuant software (Molecular Dynamics). For each gene analyzed, a cycle curve experiment was performed and the optimal number of PCR cycles for the quantitative analysis was chosen within the linear range of amplification. The primers (forward and reverse, given in the 5' to 3' orientation) and the number of cycles used in PCR are listed below.

Gene: Primers:

Alkaline phosphatase (ALP)

        CCCAAAGGCTTCTTCTTGC and

        GCCTGGTAGTTGTTGTGAG, 30 cycles

Msx2:

        CGCCTCGGTCAAGTCGGAA and

        GCCCGCTCTGCTAGTGACA, 31 cycles

Parathyroid hormone receptor (PTHR):

        ACCCCGAGTCTAAAGAGAAC and

        GCCTTTGTGGTTGAAGTCAT, 28 cycles

Osteocalcin (OCN):

        GGGCAATAAGGTAGTGAACAG and

        GCAGCACAGGTCCTAAATAGT, 28 cycles

Cbfa1(α/m and ε):

        ATGCTTCATTCGCCTCAC and

        CTCACGTCGCTCATCTTG, 29 cycles

Osteopontin (OPN):

        CACAAGCAGACACTTTCACTC and

        GAATGCTCAAGTCTGTGTGTT , 23 cycles

Osteonectin (ONC):

        CCCTGCCAGAACCATCATTG and

        TTGCATGGTCCGATGTAGTC, 23 cycles

Collagen 1α I (Col1a):

        CCCTGCCTGCTTCGTGTAAA and

        CCAAAGTCCATGTGAAATTATC, 22 cycles

18S ribosomal subunit RNA (18S rRNA):

        CCTGGATACCGCAGCTAGGA and

        GCGGCGCAATACGAATGCCCC, 12 cycles

Glyceraldehyde 3-phosphate dehydrogenase (GAPDH):

        CTGCACCACCAACTGCTTAG and

        AGATCCACGACGGACACATT, 19 cycles

SMAD1:

        TGCTGGTGGATGGTTTCACA and

        TGTCGCCTGGTGTTTTCAATA , 29 cycles

SMAD6:

GCAACCCCTACCACTTCAG and

GCCTCGGTTTCAGTGTAAGA, 28 cycles

JunB:

CAGCCTTTCTATCACGACGA and

GGTGGGTTTCAGGAGTTTGT, 31 cycles

Id2:

CCGATGAGTCTGCTCTACAA and

CCGTGTTCAGGGTGGTCAG, 27 cycles

Dlx2:

AAACCACGCACCATCTACTC and

TCGCCGCTTTTCCACATCTT, 31 cycles

Fra-1:

ACCGCCCAGCAGCAGAAGT and

AGGTCGGGGATAGCCAGTG, 30 cycles

Wnt6:

CCGACGCTGGAACTGCTC and

ATGGAACAGGCTTGAGTGAC, 33 cycles

TCF7:

ACTCTGCCTTCAATCTGCTC and

GGGTGTGGACTGCTGAAATG, 27 cycles

Low density lipoprotein receptor-related protein 5 (LRP5):

GCCAGTGTGTCCTCATCAAG and

ACGCTGGCAGACAAAGTAGA, 25 cycles

Transforming growth factor β1 (TGFβ1):

CCAAAGACATCTCACACAGTA and

TGCCGTACAACTCCAGTGAC, 27 cycles

Transforming growth factor β3 (TGFβ3):

CACCGCTGAATGGCTGTCT and

CATTGGGCTGAAAGGTGTGA, 26 cycles

TIEG:

TTCAGCAGCAAGGGTCACTC and

GACAGGCAAACTTCTTCTCAC, 28 cycles

Hey1:

GCCGACGAGACCGAATCAAT and

GCTGGGATGCGTAGTTGTTG, 30 cycles

FK506-binding protein 5 (FKBP5):

GCTGCCATCGTGAAAGAGAA and

TCCACGGCTTTGTTGTACTC, 29 cycles

Receptor activity-modifying protein 1 (RAMP1):

TCTGGCTGCTGCTGGCTCA and

TTTCCCCAGTCACACCATAG, 31 cycles

Hlx:

CGGGACAGTTCTTCGCATCT and

TTCGGTCTGGCTTGGTCAC, 28 cycles

Gene expression determination using high-density oligonucleotide microarrays: Total RNA
form each sample (treatment: non-stimulated, day 0; GP/AA/BMP-2, day 1 and 3; GP day 1
and 3) was extracted and analyzed on 5 oligonucleotide microarrays. The microarray
hybridization was performed using the Affymetrix protocols (REF): Affymetrix GeneChip®
Murine Genome U74Av2 arrays were used, which consist of coated glass slides with a series
of oligonucleotide probes synthesized *in situ*. These arrays contain probes for approximately
9,400 genes (~5,700 functionally characterized genes and ~ 3,700 EST clusters). Biotin-
labeled cRNA probes were generated from each sample to be analyzed, starting form 5 µg
of DNAse I-treated total cellular RNA, prepared as described above. The cRNA probes were
individually hybridized on the arrays and the signals were detected according to the
manufacturer's instructions (Affymetrix, Santa Clara, CA, USA).

Analysis of gene expression on microarrays: The hybridization data were analyzed by the
software provided by Affymetrix (MAS4.0), by Novartis Pharmacogenetics Network (NPGN)
software and Expressionist 3.0 (GeneData, Basel, Switzerland). Genes were considered as
expressed if they were classified as P (present), and not as M (marginal) or A (absent) at
least in one sample. The cut-off for classification as present was hybridization signal of 20;
all lower numbers were clamped to 20. All genes presented in detail were detected with
gene-specific probes, and not with probe sets that recognize the gene families. Genes were
selected as regulated by GP/AA/BMP-2 treatment if their expression deviated more than 2-
fold from the corresponding, time-matched control.

Example 1: Osteoblastic differentiation of MC3T3-1b cell line: cellular and molecular characterization

The instability of osteoblastic phenotype in cell lines in culture is a known phenomenon, leading to divergent properties of an apparently identical cell line. Murine pre-osteoblastic MC3T3-E1 cell line is a very good model of osteoblast differentiation, reproducing in vitro a sequence of several events occurring in vivo (Quarles et al., 1992, J. Bone Miner Res; 7:683-692). In order to obtain a cell line that differentiates in vitro in a fast and efficacious manner, we transfected MC3T3-E1 parental cells with a Cbfa1-dependent reporter gene and selected a clone of MC3T3-E1 cells that responds well to osteogenic stimulus (10 mM GP, 50 μM AA and 200 ng/ml BMP-2). This clone was named MC3T3-1b and characterized with respect to cellular phenotype and molecular markers of differentiation. In response to osteogenic stimulus, MC3T3-1b cell line showed a strong increase in alkaline phosphatase activity at day 3, as documented by cytochemical staining. Furthermore, this cell line produced bone nodules in culture and mineralized them by day 14, as shown by Alizarin Red S staining. After several more days, the number of nodules increased greatly, covering more than 50 % of well area. This result showed that in this cell line a whole differentiation process is reproduced within 2 weeks, which is very fast and efficient. Previously, we and others noticed that with other MC3T3-E1 clones it is necessary to maintain cultures for 3-5 weeks to obtain mineralized bone nodules, and the whole process was often inefficient (Quarles et al., 1992, as above). Thus, MC3T3-1b cells exhibit expected cellular properties for the study of osteoblast differentiation process.

Next, we analyzed messenger ribonucleic acid (mRNA) expression of a number of molecular markers of osteoblast differentiation by radioactive quantitative reverse transcription-polymerase chain reaction (rqRT-PCR). MC3T3-1b cells were stimulated with osteogenic stimulus for 1 and 3 days and compared with non-stimulated time-matched controls. Eight markers of osteoblast differentiation were analyzed: alkaline phosphatase (ALP), transcription factors Msx2 and Cbfa1, parathyroid hormone receptor (PTHR), and extracellular matrix proteins osteocalcin (OCN), osteopontin (OPN), osteonectin (ONC) and collagen Iα1 (Col Iα1). The optimal primers were designed and quantitative conditions were optimized in cycle curve rqRT-PCR experiments for each marker gene (see Methods above). In response to the osteogenic stimulus, mRNA levels of five osteoblast markers were up-regulated in MC3T3-1b cells. ALP was strongly induced already at day 1 with even higher increase at day 3. Msx2 was transiently up-regulated at day 1 and Cbfa1 was induced at day 3. PTHR and OCN, late markers of osteoblast differentiation, were strongly induced at day 3.

For three osteoblast markers, OPN, ONC and Col Iα1, high levels of expression were detected already in non-stimulated conditions and they did not change upon treatment. As MC3T3-1b cells are already committed to the osteoblast phenotype, high level of these extracellular matrix proteins may be expected. As a normalization control, mRNA levels of two housekeeping genes analyzed: 18S ribosomal RNA (18SrRNA) and glyceraldehyde-3 phosphate dehydrogenase (GAPDH). Their expression was similar in all conditions.

Example 2: Gene expression profiling by DNA microarray analysis: comparison of osteoblast marker expression by rqRT-PCR and microarrays:

After selection of an appropriate system of in vitro osteoblast differentiation, we set out to perform genome-wide analyses of gene expression during osteoblast differentiation. MC3T3-1b cell line was stimulated with osteogenic stimulus, total RNA was extracted at days 0, 1 and 3 and analyzed by Affymetrix GeneChip microarray U74. This oligonucleotide array represents about 10,000 genes, comprising genes with known function and ESTs.

As a validation step in the microarray data analysis, hybridization values obtained for osteoblast marker genes were compared with the data obtained by rqRT-PCR. The GeneChip contained oligonucleotide probes for seven of eight osteoblast markers and for one of two housekeeping genes analysed by rqRT-PCR. For these seven markers and one housekeeping gene, the same trend and a similar fold regulation was observed with two different methods. Fold up-regulation differed mostly for the genes that were up-regulated from the background to very high levels (PTHR, OCN). This is expected due to the inaccuracy of background measurement. There was a tendency to obtain smaller fold changes by microarray analysis. Strong and reliable hybridization microarray signals were obtained even for the genes with very low level of expression, indicating a high sensitivity of microarray detection in our experiments. We concluded that microarray data obtained can provide reliable information about gene expression profiles, even for the very low expressed genes like transcription factors.

Regulated expression of 382 known genes and 283 ESTs

To get an insight into transcriptional events involved in the osteoblast differentiation process, we investigated genes whose expression levels changed upon treatment with osteogenic stimulus. The levels of gene expression in treated samples was compared with time-matched non-stimulated controls. A 2-fold up- or down-regulation was taken as a selection threshold. We detected 382 genes with known function and 283 ESTs, whose expression

changed at least 2-fold at one or both time points post-stimulation (day 1 or 3 or both). In further analyses, we focused on the genes with known function.

The functional analysis of 382 regulated genes led us to define ten large groups of regulated genes: 1) Matrix Proteins and Adhesion (37); 2) Growth Factors (26); 3) Receptors (31); 4) Cytoskeleton (12); 5) Signaling (25); 6) Kinases (16); 7) Transcription Factors (51); 8) Cell cycle and apoptosis (20); 9) DNA replication (22); and 10) Others (142) (Table 1). In Table 1, within each of these groups, the genes were sorted from high to low degree of regulation, including both days 1 and 3. Most of these genes were not analyzed further and will not be discussed in detail, but they provided a complete picture of transcriptional events during osteoblast differentiation in our model system.

A large number of regulated genes (37) belonged to the group related to Matrix Proteins and Adhesion (Table 1). This is consistent with osteoblasts being adherent cells responsible for production of large amounts of bone matrix. Down-regulation of many adhesion molecules and up-regulation of others suggested that osteoblasts change their adhesion properties during differentiation. Most prominent up-regulated genes were bone sialoprotein, osteocalcin, osteomodulin and keratoepithelin (3.5-13-fold). Among down-regulated genes were cadherin 11 (osteoblast-specific cadherin), osteoglycin, laminin, thrombospondin, vascular cell adhesion molecule-1 (VCAM-1) and matrix metalloproteinase-13 (MMP-13). This pattern fits the expected changes, but also reveals some new aspects, especially among down-regulated genes.

The Growth Factors group contained genes expected to be regulated in osteoblasts, but also some novel genes (Table 1). Some of the expected genes are the known modulators of osteoblast proliferation: PDGFα, VEGF, FGF, IGF binding proteins, PTH-RP (Canalis and Rydziel, 1996, Chapter 44, 619-626, Academic Press Inc; Hurley and Florkiewicz, 1996, Chapter 45, 627-645, Academic Press Inc.; Conover, 1996, Chapter 43, 607-618, Principles of Bone Biology; Swarthout et al., 2002, Gene, 282:1-17). Some of the novel regulated genes were those of extracellular factors TGF-β and Wnt families (TGF-β1-3 and BMP-4, and Wnt1, 6, and 10a) and of their extracellular regulators (BMP inhibitor Gremlin). Although their connection to bone physiology is known (TGF-β, Kim and Ballock, 1993, J Cell Biochem. 77:169-78) or only recently suggested (Wnt, Gong et al., 2001, Cell, 107:513-523), their expression by osteoblasts and regulation during differentiation has not yet been reported. Wnt 10a is the most prominent stimulated gene, with 6.8-fold up-regulation, while Gremlin is the most prominently inhibited gene, with 0.12 relative to control. Interestingly, many members of the corresponding signaling pathways of TGF-β and Wnt were also

regulated, as will be shown and discussed below. Furthermore, M-CSF, small inducible cytokine A7 and PTH-RP, all of which can stimulate differentiation or function of bone-resorbing osteoclasts, were down-regulated, suggesting reduced ability of differentiating osteoblasts to stimulate osteoclasts.

In the Receptors group, up-regulated genes included those coding for the receptors of many factors implicated in osteoblast differentiation or function: PTH receptor PTHR, LIF receptor LIFR, leptin receptor LEPR, prostaglandin F receptor PTGFR, FGF receptor FGFR2, nuclear orphan receptor RORα, urokinase receptor PLAUR, Neuropilin 1/VEGF165 receptor (Swarthout et al., 2002, Gene, 282:1-17; Malaval and Aubin, 2001, J Cell Biochem, 81(S36):63-70; Gordeladze et al., 2001, Curr Pharm Des, 7:275-290; Pilbeam et al., 1996, Chapter 51, 715-728, Academic Press Inc.; Wilkie, 1997 Hum Mol Genet, 6:1647-56; Meyer et al., 1999, PNAS USA, 97:9197-202; Allan et al., 1995Clin Orthop, 313:54-63; Deckers et al., 2000, Endocrinology, 141:1667-74) (Table 1). Some of the up-regulated receptors have not been reported previously to play a role in osteoblasts, such as ephrin receptors (EPHA2, EPHB2) and somatostatin receptors (SSTR4, SSTR2), both of which were up-regulated in our system 3-5-fold. The highest up-regulated receptor genes (>30-fold) were those for PTH receptor (PTHR) and a co-receptor for calcitonin receptor-like receptor RAMP1. While up-regulation of PTHR as an accepted osteoblast marker was expected, up-regulation of co-receptor RAMP1 was surprising. Interestingly, osteoprotegerin (OPG), a decoy receptor for RANKL, a main cytokine in osteoclastogenesis, was up-regulated, suggesting that differentiating osteoblasts have reduced ability to stimulate osteoclast formation. Down-regulated genes included secreted form of interleukin-4 receptor IL4R, vascular endothelial growth factor receptor VEGFR2, oncostatin receptor OSMR, platelet-derived growth factor receptor PDGFRα, low density lipoprotein receptor-related protein 5 LRP5, and prostaglandin E receptor 1 PTGER1 (Ura et al., 2000, Endocr J, 47:293-302; Hurley and Florkiewicz R, 1996, Chapter 45, 627-645, Principles of Bone Biology, Academic Press Inc.; Jay et al., 1996, Endocrinology, 137:1151-8; Canalis and Rydziel, 1996, Chapter 44, 619-626, Principles of Bone Biology, Academic Press Inc.; Pilbeam et al., 1996, Chapter 51, 715-728, Principles of Bone Biology, Academic Press Inc.). Most highly inhibited genes (0.11-0.13 of control) were those for the chemokine orphan receptor 1 (RDC1) and prostaglandin E2 receptor 1 (EP1). Reduced levels of mRNA for these receptors suggests that they perform a function in pre-osteoblasts and that their signals need to be reduced for osteoblast differentiation to proceed. This is easily understood for PDGFRα, which is not necessary any more after osteoblasts have completed their proliferation phase and started to differentiate.

The Cytoskeleton, Signaling Molecules and Kinases groups are not mentioned in detail, because the connection of regulated genes with bone physiology is not clear at present. Nevertheless, some of these genes would also deserve a closer investigation in the future. We will only mention an interesting case of specific gene regulation within one family: the cytosolic protease calpain 6 was up-regulated 4.1-fold, while calpain 5 was expressed at 0.5-fold relative to the control.

The Transcription Factors group is the largest and contains many interesting regulated genes. Transcription factors are generally weakly expressed; however, we detected about 50 regulated transcription factors, all with a reliable signal intensity (Table 1). Furthermore, we analyzed the regulation of 10 transcription factor genes by rqRT-PCR and independently confirmed the regulation observed by microarray analysis (see below). A large number of regulated transcription factors suggested that their orchestrated regulation is key to the osteoblast differentiation. Many of them have not yet been linked to osteoblasts or bone, and for simplicity reasons we will focus on the up-regulated genes and those forming functionally-linked subgroups. They belong to the following structural subgroups: homeobox (Msx2, HOXC9, Dlx2, and Hlx), Zn finger (Zac1, BRCA1, Egr2/Krox20, PML), and helix-loop-helix (Hey1, Id1-3, CLOCK). Functionally, they are on the signaling pathways of TGFβ family, Wnt extracellular factors and the Notch receptor. The genes belonging to these three signaling pathways are mentioned below.

The regulation of many genes involved in cell cycle and DNA replication is consistent with our observation that the cells, although confluent prior to stimulation, still continue to proliferate to a small degree up to 3 days post-stimulation with osteogenic stimulus. Finally, the genes in the group Others comprise those whose connection to bone physiology is presently unclear and which did not belong to any of the above mentioned large gene groups. In Table 1, we show the most interesting members of this group.

In the rest of this study, we concentrated on the analysis of the genes belonging to the functionally most interesting groups.

Example 3: Genes coding for proteins with established function in osteoblast differentiation and regulation of osteoclast formation

We first evaluated the expression of genes with an established function in osteoblast differentiation and osteoclast formation, two main processes in osteoblastic cells (Table 2). The identified genes were grouped into following categories: 1) BMP signaling pathway; 2) BMP target genes; 3) Osteoblast differentiation (including osteoblast markers); and 4)

Stimulation of osteoclastogenesis. It should be noted that although the function of these genes has been linked to bone cells, for many of them, this type of regulation during osteoblast differentiation was not previously reported.

Major components of BMP-2 signaling pathway, SMAD molecules, were shown to be up-regulated 2-3-fold. The mRNA expression of SMAD 6 and 7, the inhibitory SMADs, was transiently up-regulated at day 1 (Table 2). This probably represents a feed-back mechanism for down-regulation of BMP signaling and is in agreement with a recent study (Locklin at al, 2001, J Bone Miner Res, 16:2192-204). SMAD1, BMP-specific SMAD, was up-regulated at day 3, showing that positive BMP signaling components are stimulated as well. Gremlin, the secreted BMP-2 antagonist, was strongly down-regulated by osteogenic stimulus at both days 1 and 3, suggesting further regulatory mechanism for stimulation of BMP-signaling. Some genes that were previously shown to be BMP-2 target genes in osteoblasts (Locklin at al, 2001, J Bone Miner Res, 16:2192-204) were also regulated in our study. JunB, AP-1 complex component that is involved in osteoblast differentiation (Jochum et al., 2001, Oncogene, 20:2401-12), was strongly up-regulated at both days 1 and 3. Id group of HLH transcriptional inhibitors (Lopez-Rovira et al., 2002, J Biol Chem, 277:3176-85). Id1, 2 and 3 were strongly up-regulated, while Id4 was down-regulated. The homeobox transcription factor Dlx2, known to be important for craniofacial patterning and morphogenesis during embryonic development (Xu et al., 2001, DNA Cell Biol, 20:359-65), was transiently up-regulated at day 1.

Other genes with function in osteoblasts were also regulated. Fra-1 (FosL-1), a transcription factor in focus of bone biology, was shown to be important both for bone formation in vivo and for osteoclast differentiation in vitro (Matsuo et al., 2000, Nat Genet, 24:184-7, Jochum et al., 2001, Oncogene, 20:2401-12). Its mechanism of action in osteoblasts in vitro has not been investigated. We detected a strong and transient up-regulation of Fra-1 (3.4-fold). Transcription factor Msx1 was shown to be down-regulated upon terminal differentiation of chondrocytes, muscle cells, and most recently, odontoblastic cells (Blin-Wakkach, 2001, PNAS USA, 98:7336-41 and references therein). We detected a strong down-regulation of Msx1 (0.25-fold relative to control). Leptin has been associated with bone physiology and the prevailing hypothesis suggests brain as its site of action (Ducy, 2000, Science, 289:1501-4). However, in-house studies have shown up-regulation of the leptin receptor during osteogenic differentiation of human mesenchymal cells. We confirmed the up-regulation of leptin receptor also in our MC3T3-1b cell system (5-fold). Up-regulation of retinoic acid receptor related protein alpha (RORα) during osteogenic differentiation of human mesenchymal cells

has also been shown in-house and we also detected 2.6-fold up-regulation. Most recent data suggested that a mutated low density lipoprotein receptor-related protein 5 (LRP5) is a high bone mass gene in humans, while other mutations lead to osteoporosis-pseudoglioma syndrome (Gong et al., 2001, Cell, 107:513-523; Little et al., 2002, Am J Hum Genet, 70:11-9; Boyden et al., 2002, N Engl J Med, 346:1513-21). In our system, LRP5 is down-regulated upon treatment with osteogenic stimulus at both days 1 and 3. BMP-4, a closely related gene to BMP-2 and strong osteogenic agent (Yamaguchi et al., 2000, Endocr Rev, 21:393-411), is also down-regulated.

Cells of osteoblastic lineage are stimulators of osteoclast formation, mainly via up-regulation of cytokines M-CSF and RANKL (reviewed in Takahashi et al., 1999, Biochem Biophys Res Commun, 256:449-55). Upon treatment with osteogenic stimulus, we detected up-regulation of OPG, an inhibitor of RANKL by 3.8-fold. M-CSF, PTH-RP, and small inducible cytokine A7, all of which can stimulate osteoclast formation and/or function are down-regulated. In agreement with that, the transcription factor Egr1, a negative regulator of M-CSF (Cenci et al., 2000, J Clin Invest, 105: 1279-87), was up-regulated by 3.6-fold. RANKL was not expressed by MC3T3-1b cells before nor after stimulation with osteogenic stimulus. We conclude that MC3T3-1b cells express and regulate molecules involved in osteoclast formation. The direction of regulation of these molecules suggests that the ability for the support of osteoclastogenesis is reduced during osteoblastic differentiation.

Example 4: Activation of TGFβ signaling pathway

Three members of transforming growth factor β family (TGFβ) were regulated (TGFβ1, 4-fold up-regulation at days 1 and 3, TGFβ2, 2-fold down-regulation at day 3, and TGFβ3, 2-fold up-regulation at day 1 (Table 3). TGFβ family members are potent modulators of osteoblast differentiation process (reviewed in Kim and Ballock, 1993, Cellular and Molecular Biology of bone, Academic Press, 98-122), but their regulation by osteogenic stimulus containing BMP-2 has not been reported yet. Interestingly, two family members are up-regulated and one is down-regulated. Since MC3T3-1b cells are responsive to TGFβ, we investigated the expression of the components from TGFβ signaling pathway on microarrays. Type I TGFβ receptor was expressed, but not regulated, while signal for type II receptor was absent, suggesting a low expression level. SMAD2 probe was not present on the chip, while SMAD3 was not detected. From the Western blot data, we know that SMAD2 is present and phosphorylated in response to TGFβ-1. The inhibitory SMAD6 and 7 were transiently up-regulated, as mentioned above. The activation of TGFβ pathway by

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stimulation with the osteogenic stimulus was confirmed by regulation of several TGFβ target
genes: transcriptional repressor TIEG (Hefferan et al., 2000, J Cell Biochem, 78:380-90);
extracellular matrix protein tenascin C (Mackie et al., 1998, Bone 22: 301-7), adhesion
molecule kerato-epithelin (Kim et al, 2000, J Cell Biochem, 77:169-78), and somatostatin
receptor type 2 (SSTR2) (Puente et al., 2001, J Biol Chem, 276:13461-8). We conclude that
stimulation of pre-osteoblasts with a mixture of GP, AA and BMP-2 induces production of
TGFβ1 and 3, which are in turn capable of inducing TGFβ signaling cascade. It would be
interesting to determine which component of the osteogenic stimulus leads to the induction
of TGFβ1 and 3. These results shed a new light on the interplay between different growth
factors in osteoblastic differentiation.

Example 5: Activation of Wnt signaling pathway

Three members of Wnt family of secreted proteins was up-regulated: Wnt1, 2-fold, Wnt6, 4-
fold, and Wnt10a, 5-fold, (Table 4). Wnt proteins are known to control multiple
developmental processes (reviewed in Wodarz and Nusse, 1998, Annu Rev Cell Dev Biol,
14:59-88), including limb patterning and joint formation. Very recently they came into a focus
of bone biology as it was shown that a co-receptor for Wnt, the low density lipoprotein
related protein 5 (LRP5), was responsible for two striking human skeletal phenotypes,
osteoporosis-pseudoglioma syndrome and high bone mass (Gong et al., 2001, Cell,
107:513-23, ; Little et al., 2002, Am J Hum Genet, 70:11-9). For that reason, we investigated
the expression of whole Wnt family and its pathway components. Except for the regulated
Wnt family members mentioned above, the expression of Wnt 5b and Wnt 10b were also
expressed, but not regulated more than 2-fold. Other genes from Wnt 16-member family
were not expressed.

Frizzled are a family of seven transmembrane – G protein coupled receptors to which Wnt
proteins bind (reviewed in Wodarz and Nusse, 1998, Annu Rev Cell Dev Biol, 14:59-88).
From 8 members of the family, higher expression was detected for Frizzled 1, 2, and 8
(Table 4). Secreted Frizzled-related sequence proteins (sFRPs) can act as decoy receptors
for Wnt, and thus inhibit Wnt signaling. Very high expression of sFRP2 was detected, but not
of sFRP1 and 4. sFRP2 was down-regulated at day 3, suggesting stimulation of Wnt
signaling. The expression of LRP5 and its homologues was also analyzed. Low density
lipoprotein receptor (LDLR) and LRP1 are strongly expressed and LRP1 was 3-fold down-
regulated at day 3. LRP5 and LRP6 were also expressed and LRP5 was down-regulated to a
background level at days 1 and 3. However, LRP5 mRNA was still detectable by rqRT-PCR

(see below). The significance of this down-regulation is not clear at present. Possibly, its high expression is necessary very early in osteoblastic differentiation. Dickkopf is a family of proteins that extracellularly bind and regulate Wnt signaling via LRPs (Bafico et al., 2001, Nat Cell Biol, 3:683-686). Transfection experiments suggested that a Dickkopf family member constitutively inhibits LRP5 signaling, which is prevented by LRP5 mutation found in high bone mass gene (Boyden et al., 2002, N Engl J Med, 346:1513-21). However, identity of endogenous osteoblast Dickkopf is not known. We found that Dickkopf-3 was the only family member expressed at high levels in MC3T3-1b cells (Table 4). The other components of the Wnt signaling pathway, such as β-catenin, axin, APC, LEF1 and TCF4 were also expressed. The transcription factors mediating Wnt signaling, LEF1 and TCF4, were both up-regulated. Finally, the Wnt pathway target gene TCF7 was strongly up-regulated (Table 4).

We concluded that a mixture of GP, AA and BMP-2 induces expression of several Wnt family members. The Wnt receptors and the signaling machinery are present and up-regulation of Wnt target genes indicates that the expressed Wnt are active. This is a novel aspect of osteoblast differentiation process.

Example 6: Activation of Notch signaling pathway

The transcription factor Hey1 was strongly up-regulated: 8.5-fold at day1, and 3.3-fold at day 3 (Table 5). Hey1 is a member of a recently described family of basic helix-loop-helix (bHLH) transcription factors, related to the hairy and enhancer of split (HES). The family consists of three members: Hey1, Hey2 and HeyL (Leimeisteret et al., 1999, Mech Dev, 85:173-7). Together with HES, they are thought to be direct targets of the Notch signaling pathway (Iso et al., 2001, Mol Cell Biol,21:6071-9; Iso et al, 2002, J Biol Chem, 277:6598-607). Notch signaling is an evolutionarily conserved mechanism used by metazoans to control cell fates through local cell interactions (Artavanis-Tsakonas et al., 1999, Science, 284:770-6).

Strong up-regulation of Hey1 transcription factor suggested that Notch pathway is activated in MC3T3-1b cells during osteogenic differentiation. Therefore, we examined the expression of Notch ligands, Notch itself as a receptor, as well as factors influencing Notch activity and the target genes (Table 5). The Notch ligands Delta and Jagged, for which the probes were present on the microarray, were not detected. Nevertheless, other ligands are described for which the probes were not present. Furthermore, Notch pathway can be activated also without these ligands, through a proteolytic degradation pathway (Mumm and Kopan, 2000, Dev Biol, 228:151-165). Other factors involved in Notch processing, trafficking and regulation

were expressed, such as protease ADAM10, presenilin1 and 2, and Lunatic and Radical fringe homologues (Greenwald, 1998, Genes Dev, 12:1751-62). ADAM10 expression was up-regulated 2-fold at day 3, indicating a possible activation of the Notch pathway. Notch receptor and ADAM10 are both expressed in bone (Dallas et al., 1999, Bone, 25:9-15). We concluded that activation of the Notch pathway is an exciting novel aspect of osteoblast differentiation process.

Example 6a - BMP-2 induction of Hey1 gene:

Induction of Hey1 by BMP-2 in MC3T3 cells. MC3T3 cells are cultured in the absence (medium) or presence of GP, AA, and/or BMP-2 for 1, 2, 3 and 4 days. Total RNA is extracted and analyzed for expression of Hey1 and the control 18S ribosomal RNA by rqRT-PCR. The radioactive PCR products are analyzed by polyacrylamide gel electrophoresis and imaging by PhosphorImager. Gel images obtained by PhoisphorImager are shown. B: Induction of Hey1 by BMP-2 in human mesenchimal steam cells (MSC) and C2C12 cell line. Data obtained by microarray analysis. Human mesenchymal stem cells (MSC): Affymetrix array HG-U133A, probe set number 218839_at, sequence accession number NM_012258; C2C12: Affymetrix array MG-U74Av2, probe set number 95671_at, sequence accession number AJ243895.

Result: Hey1 is induced by BMP-2 in murine MC3T3-pre-osteoblastic cells, as well as in dually-potent murine C2C12 cells, and human mesenchymal stem cells.

Example 6b - siRNA mediated inhibition of Hey1 induction:

MC3T3 cells are plated on 6-well plates (0.5×10^5 cells/well in 2 ml of medium). After 24h, siRNA is performed in a total volume of 1 ml using Oligofectamine (Life Technologies), according to the manufacturer's instructions. siRNA concentration is 0.1 µM, and the Oligofectamine amount 4 µl/well. Transfection is stopped after 4 h by adding 0.5 ml of medium containing 30% of serum, and the osteogenic stimulus. RNA is isolated after 1, 2, 3 or 4 days. For Alizarin Red S staining, medium containing 10% FCS and the osteogenic stimulus is changed twice weekly. Staining is performed after 17 days. siRNA sequences: Hey siRNA, sense strand GCTAGAAAAAGCTGAGATC, dTdT overhangs IE-HPLC purified (Xeragon Inc.). Control siRNA, sense strand AGAAGGAGCGGAATCCTCG, dTdT overhang.

A | Day 1 | Day 2 | Day 3 | Day 4

(lanes: untreat, treat, untreat+mock, treat+mock, Hey1siRNA, Control siRNA, untreat+mock, treat+mock, Hey1siRNA, Control siRNA, untreat+mock, treat+mock, Hey1siRNA, Control siRNA, untreat+mock, treat+mock, Hey1siRNA, Control siRNA, RT-)

Inhibition: 86%   76%   71%   -6%

B | Day 1 | Day 2

(lanes: untreat+mock, treat+mock, Hey1siRNA, control iRNA, untreat+mock, treat+mock, Hey1siRNA, control siRNA, RT-)

ALP
Osx
Id2
Dlx2
Fra1

C   Mineralization

Untreat +mock   Treat +mock

Hey1 siRNA   Control siRNA

Transient inhibition of BMP-2 induced Hey1 expression by Hey1 specific siRNA. MC3T3 cells are either not transfected (untreat, treat), mock transfected (untreat+mock, treat+mock) or transfected with Hey1 specific siRNA (He1siRNA) or ineffective, control siRNA (control siRNA). After 4 h of transfection, cells are either left untreated (untreat, untreat+mock) or are treated with GP/AA/BMP-2 (treat, treat+mock, Hey1siRNA, control siRNA). Total RNA is

isolated after 1, 2, 3 or 4 days and gene expression is analyzed by rqRT-PCR. B: No effect of Hey1 inhibition on the expression of early BMP-2-induced osteoblast genes by rqRT-PCR analysis. C: Transient inhibition of Hey1 induction affects mineralization process. MC3T3 cells are either mock transfected (untreat+mock, treat+mock), transfected with Hey1 specific siRNS (Hey1siRNA) or with ineffective, control siRNA (control siRNA). After 4 h of transfection, cells are either left untreated (untreat) or treated with GP/AA/BMP-2 (treat+mock, Hey1siRNA, control siRNA). Alizarin Red S staining is done after 17 days. Result: Hey1 expression can be inhibited by Hey1 siRNA, leading to little change in early gene expression, but affecting the long-term mineralization process.

Example 6c - Cbfa1 transcriptional activity is inhibited by Hey1:

Cbfa1 and Hey1 cDNA are cloned into expression vector pcDNA3.1 (+) and used in a luciferase reporter assay. As a reporter vector OG2luc plasmid was used, consisting of minimal osteocalcin promoter and eight OSE2 Cbfa1 binding sites in front of the luciferase gene. Cbfa1 binding sites were wild type (8XOSE2 wt), or mutated to abolish Cbfa1 binding (8XOSE2 mut). Verification of Cbfa1 and Hey1 overexpression by rqRT-PCR.

Result: Cbfa1 induced 8XOSE2-dependent luciferase activity, and this effect is inhibited by coexpression of Hey1.

Example 6d - Assay with Hey1 promoter reporter gene (to obtain antagonists or agonists to Hey1)

a) Generation of osteoclastic cell line stably expressing Hey1 promoter luciferase

Hey1 promoter is cloned into a luciferase reporter gene vector (Rapid ResponseTM reporter vectors from Promega, Madison, WI, USA, or similar). A variety of pre-osteoblastic or pluripotent mouse cell lines is used as hosts for generation of stable cell lines: MC3T3, C2C12, CH310T1/2. Using standard techniques with antibiotics, a stable clone is selected. Further selection are based on best fold and potency of luciferase induction by BMP-2 (800 ng/ml).

b) Cell culture for high throughput screening

All cell lines are incubated at 37°C in humidified atmosphere under 5% CO_2. C2C12 cells are routinely cultivated in DMEM HG medium supplemented with 10% FBS, 2 mM glutamine, 50 µg/ml gentamicin, 10 mM Hepes and 0.5 mg/ml G418. MC3T3 and CH310T1/2 cells are maintained in α-MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 IU/ml penicillin,

100 μg/ml streptomycin and 0.75 mg/ml G418. For the reporter gene experiments, all the clones are grown in the absence of G418.

BMP-2 is stored as 500 μg/ml stock solution in water at -80°C and after thawing at 4°C. Final concentration is 800 ng/ml. For high controls with BMP-2, 500 μg/ml BMP-2 is diluted 1:250 in culture medium containing 1.25 % DMSO and 20 μl were added to 30 μl of cell suspension in wells on 384-well plate. Final concentration was 800 ng/ml of BMP-2. Low controls are done with medium containing 1.25 % DMSO (BMP-2 controls) or with medium containing 0.63 % DMSO (TGF-b1 controls). Twenty μl is added to 30 μl of cell suspension in wells on a 384-well plate.

c) Reporter gene assay for high throughput screening

The cells in triple flasks are washed with 30 ml phosphate-buffered saline (PBS). Trypsin/EDTA solution is added (15 ml) and incubated with cells for 1-2 min at 37°C. The culture medium is added (15 ml) and the cells are collected by centrifugation. The cells are counted and diluted to 6.7×10^3 cells/ml (C2C12) or 1.78×10^4 cells/ml (MC3T3 and CH310T1/2). Fifty μl of this suspension is added to wells in Greiner 384-well plates with the Multidrop machine in sterile conditions. The cell suspension is constantly mixed during distribution with the Multidrop. The Multidrop cassette is maintained sterile with 70 % ethanol. The final cell number per well is 333 (C2C12-15a) and 888 (MC3T3 and CH310T1/2). The cells are grown for 3 days until confluency at 37°C in a tissue culture incubator. The compounds or reference stimulators (BMP-2) are added to the confluent cells using Multidrop and Cybiwell. The incubation is continued for another 24 h.

Luciferase activity is measured as follows: plates are brought from 37°C to the room temperature and 25 μl of SteadyGlo reagent is added to each well. The plates are incubated for 60 min at room temperature and luminescence is measured at Viewlux luminometer (60 s measurement time, binning factor 2, medium speed, medium gain).

d) Criteria for hit identification in high throughput screen

Compounds inducing significant activation or reduction (agonists and antagonists) of luciferase (usually >2-fold induction) are taken as a first set of hits. The compounds are confirmed in dose response curves with at least 9 concentration points. Compounds producing regular sigmoidal curves are taken as first priority hits.

Note: Agonists and Antagonists of Hey-1 are interesting for bone loss diseases as antagonists stimulate osteoblast differentiation (direct implication); and Agonists keep

mesenchymal stem cells in their pluripotent stage, thus generating higher numbers of cells that can be stimulated to differentiate with endogenous factors (indirect implication).

Example 7: Other interesting regulated genes

Several additional up-regulated genes not belonging to the above groups will be mentioned: FKBP5, RAMP1, and three enzymes from the cholesterol biosynthesis pathway. The criteria for their selection were: very strong up-regulation, up-regulation of several genes from the same pathway, and a relationship with very recent findings in bone biology. These genes are already included in the Table 1, either as a part of big superfamilies, or in the group of Other genes.

FKBP5 belongs to the family of immunophilins, a group of intracellular receptors for immunosuppresive drug FK506. Upon binding of FK506, FKBPs inhibit Ca^{2+}-dependent protease calcineurin. FKBPs have intrinsic peptidyl/prolyl cis/trans isomerase activity (reviewed in Gothel and Marahiel, 1999, Cell Mol Life Sci, 55:423-36). Recent report showed that FK506 enhances osteoblast differentiation process in mesenchymal cells (Tang et al., 2002, Cell Biol Int, 26:75-84), suggesting a connection between FKBPs and osteoblasts. We observed 7-fold increase in the expression of FKBP5 at day 3 (Table 1, Others).

Together with the calcitonin receptor-like receptor, the receptor activity-modifying protein 1 (RAMP1) forms the functional receptor for calcitonin gene-related peptide, (CRLR) (McLatchie et al., 1998, Nature 393:333-9). Striking up-regulation of RAMP1 by 30-fold was detected at day 3 (Table 1, Receptors). The change in RAMP1 expression during osteoblast differentiation is a novel finding.

Three genes coding for enzymes involved in cholesterol biosynthesis were up-regulated. Rate-limiting enzyme HMG CoA reductase is up-regulated 11-fold, suggesting importance of cholesterol or some of its precursors in the osteoblast differentiation process (Table 1, Others). Sterol-C5-desaturase and dehydrocholesterol reductase were up-regulated 3-fold. The involvement of mevalonate pathway of cholesterol production and geranylgeranyl modification of proteins has been identified as a target of bisphosphonates in the bone resorbing cells osteoclasts (Coxon et al., 2000, J Bone Miner Res, 15:1467-1476). In osteoblastic cells, the mevalonate pathway was implicated in the mode of action of statins, which were in some systems shown to have a bone anabolic effects (Garrett et al., 2001, Curr Pharm Des, 7:65-70). Furthermore, the identification of the mutated LRP5 as high bone

mass gene (Little et al., 2002, Am J Hum Genet, 70:11-9) raised the interest in other related low density lipoprotein receptor genes and their ligands.

Example 8: Confirmation of the gene expression profiles by rqRT-PCR

We have independently confirmed observed regulation of 16 genes by rqRT-PCR, 10 of them coding for transcription factors. From most groups discussed above, we selected few representatives: for BMP-2 signaling pathway -SMAD1 and 6, for BMP-regulated genes - JunB, Id2 and Dlx2, for osteoblast differentiation related genes - Fra1, for TGFβ signaling pathway - TGFβ1, TGFβ3 and TIEG, for Wnt signaling pathway - Wnt6, TCF7 and LRP5, for Notch signaling pathway - Hey1, for other interesting genes - FKBP5, RAMP1 and Hlx (see Table 1). For all the genes analyzed, the expression profiles obtained by rqRT-PCR closely corresponded to the profiles obtained by microarray analysis. Together with osteoblast markers and a housekeeping gene, the number of analyzed and confirmed genes was 24, indicating high reliability of gene regulation by microarrays.

(a)   *Table 1. Functional classification of genes regulated during osteoblastic differentiation of MC3T3-1b cells.*

Matrix Proteins and Adhesion				
Name	Description	Accession number	Relative expression level	
			Day 1	Day 3
Bone sialoprotein	Integrin binding sialoprotein	L20232	1.00	12.85
Osteocalcin	Bone gamma carboxyglutamate protein 1	L24431	1.00	10.20
Osteomodulin	Proteoglican, bone matrix protein	AB007848	0.97	4.42
TGFBI. Kerato-epithelin	Extracellular adhesion molecule,binds to collagen 1, 2 and 4. TGF-beta induced	L19932	3.49	2.05
Sprr2	Cornified cell envelope of stratified squamosus epithelia	AJ005569	1.80	3.25
Tenascin C	Extracellular matrix glycoprotein. Secreted by OB. Stimulate OB differentiation. TGF-beta induced	X56304	2.53	2.83
Vinculin	Cytoplasmic face of adhesion plaques	L18880	0.83	2.76
Periplakin	Component of the cornified envelope of keratinocytes	AF013715	0.71	2.36
Peptidase 4 / prolidase	Recycling of proline for collagen synthesis and cell growth	U51014	1.28	2.25
PG-M	Chondroitin sulfate proteoglycan 2	D45889	2.12	0.65
Pinin	Desmosome-associated protein	Y08701	1.43	2.10
SPRR2A. Small proline-rich protein 2A	Cross-linked envelope protein of keratinocytes	AJ005559	0.23	2.00
Cadherin 11 (OB-cadherin)	Calcium-dependent adhesion protein	D21253	0.84	0.49
NID. Nidogen 1 (entactin)	Basement membrane protein	L17324	0.99	0.48
VCAM-1	Vascular cell adhesion molecule 1	M84487	0.64	0.45
CHST2	Carbohydrate (chondroitin 6/keratan) sulfotransferase 2	AB011451	1.20	0.44
Lysyl oxidase-2	Extracellular copper-dependent enzyme. Initiates cross-linking of collagens and elastin	U79144	1.02	0.44
JUP. Junction plakoglobin	Common junctional plaque protein	M90365	0.44	0.54
Osteoglycin	Small leucine-rich proteoglycan. In conjunction with TGFbeta1 orTGFbeta2, induces bone formation	D31951	0.43	0.45
Glypican 4	Cell surface heparan sulfate proteoglycan	X83577	0.66	0.42
Matrilin 4	Extracellular matrix protein	AJ010984	0.41	0.43
Laminin B1	Major component of basal laminae	X05212	0.35	0.40

Thrombospondin 2	Extracellular matrix glycoprotein	L07803	0.57	0.36
ALCAM	Activated leukocyte cell adhesion molecule	L25274	0.36	0.95
Pactolus	Integrin beta subunit-like cell surface protein	AF051367	0.34	1.00
Fibulin 2	Extracellular matrix protein, binds to fibronectin	X75285	0.54	0.33
P4HA1	Post-translational formation of 4-hydroxyproline in collagens	U16162	0.33	0.47
NID2 Nidogen 2 (osteonidogen, entactin-2)	Basement membrane protein	AB017202	0.33	0.33
VCAM-1	Vascular cell adhesion molecule-1	U12884	0.50	0.31
T-VCAM-1	Vascular cell adhesion molecule-1 truncated form	U12884	0.53	0.27
Latent TGF beta binding protein 2.	Structural extracellular matrix protein for targeting TGF-beta action.	AF004874	1.21	0.26
Fibrillin 1	Major constituent of the extracellular microfibrils	L29454	0.62	0.26
Procollagen, type IV, alpha 2	Basement membrane constituent	X04647	0.64	0.26
MMP13	Collagenase-3	X66473	0.87	0.22
Procollagen, type IV, alpha 1	Basement membrane constituent	M15832	0.35	0.20
AOC3. Amine oxidase, copper containing 3	Vascular adhesion protein 1	AF078705	0.37	0.14
ENG. Endoglin	Major glycoprotein of vascular endothelium. Binds TGF-beta	X77952	0.14	0.68

Growth Factors

Name	Description	Accession number	Relative expression level	
			Day 1	Day 3
Wnt10a	Signaling molecule, secreted and associated with extracellular matrix	U61969	1.60	6.80
PPY. pancreatic polypeptide	Pancreatic hormone. synthesized in pancreatic islets of Langerhans. Regulator of pancreatic and gastrointestinal functions.	M18208	1.00	4.80
TGFbeta1. Transforming growth factor beta 1	Regulator of OB proliferation and differentiation	AJ009862	4.56	3.56
Wnt6	Signaling molecule, secreted and associated with extracellular matrix	M89800	1.74	3.85
PDGF alpha. Platelet derived growth factor, alpha	Regulator of OB proliferation and differentiation	M29464	1.04	2.70

IGFBP10. Insulin-like growth factor binding protein 10	Modulate the growth promoting effects of the IGFs	M32490	2.70	1.61
Wnt1	Signaling molecule, secreted and associated with extracellular matrix	M11943	2.30	1.00
SEMA3A. Semaphorin IIIa, collapsin I	Needed for normal patterning and growth of nerves, bones and heart	D85028	0.79	2.18
TGFbeta3	Regulator of OB proliferation and differentiation	M32745	2.10	1.12
Cardiotrophin-1	IL-6 superfamily. Binds to and activates the LIFR/gp 130 receptor complex	U18366	0.49	1.15
Mac2 (galectin-3)	Galactose- and IgE-binding lectin secreted by inflammatory macrophages and in colonic epithelium	X16834	0.46	0.94
IGFBP2. Insulin-like growth factor binding protein 2	Inhibit or stimulate the growth promoting effects of the IGFs	X81580	0.46	0.81
SCYD1 (fractalkine, neurotactin)	Small inducible cytokine subfamily D member 1. Regulating leukocyte adhesion and migration processes at the endothelium	U92565	0.63	0.45
Small inducible cytokine A7	Chemotactic factor for monocytes	X70058	0.74	0.45
IGFBP6. Insulin-like growth factor binding protein-6	Inhibits or stimulates the growth promoting effects of the IGFs	X81584	0.81	0.45
BMP-4	TGF-beta superfamily. Stimulates OB differentiation	L47480	0.44	0.77
TGFbeta2	Regulator of OB proliferation and differentiation	X57413	0.95	0.43
M-CSF	Macrophage colony-stimulating factor. Stimulates osteoclastogenesis.	M21952	0.74	0.41
VEGF- D. Vascular endothelial growth factor D	Stimulates endothelial cell proliferation. Stimulates angiogenesis	X99572	0.99	0.36
Phosphohexose Isomerase (PHI)	Ectoenzyme/exoenzyme family; glycolysis and gluconeogenesis pathways; Upon secretion-cytokine with tumor autocrine motility factor (AMF), neuroleukin (NLK) and maturation factor (MF) functions	M14220	0.36	0.64
Fibroblast growth factor 7	Keratinocyte growth factor	Z22703	0.38	0.36
Angiotensinogen	Inhibits OB differentiation (in vitro)	AF045887	0.56	0.26
Fibroblast growth factor 18	Stimulates hepatic and intestinal cell proliferation	AB004639	0.45	0.23

Parathyroid hormone-like peptide (PTHRP)	Supports osteoclastogenesis, osteolytic bone metastases and cartilage-matrix degradation during endochondral bone formation	M60057	0.23	0.18
Gremlin	Secreted. BMP antagonist	AF045801	0.12	0.15

Receptors

Name	Description	Accession number	Relative expression level	
			Day 1	Day 3
PTHR-Parathyroid hormone receptor	GPCR	X78936	2.55	33.85
RAMP1	Transports calcitonin-receptor-like receptor to the plasma membrane where it acts as a calcitonin-gene-related peptide receptor	AJ250489	1.00	30.00
LIFR-Leukemia inhibitory factor receptor	Cytokine family of receptors.	D17444	2.21	5.92
LEPR. Leptin receptor	Cytokine family of receptors.	U42467	1.00	4.95
EPHA2. Ephrin type A receptor 2	Receptor tyrosine kinase	U07634	4.85	0.96
EPHB2. Ephrin type B receptor 2	Receptor tyrosine kinase	L25890	3.95	2.30
OPG. Osteoprotegerin	Tumor necrosis factor receptor superfamily, member 11b. Inhibits OC genesis	U94331	1.27	3.84
SSTR4. Somatostatin receptor type 4	GPCR	U26176	0.42	3.26
PTGFR. Prostaglandin F receptor	GPCR	D17433	1.33	3.14
FGFR2. Fibroblast growth factor receptor 2	Receptor tyrosine kinase	M63503	1.57	2.91
SSTR2. somatostatin receptor type 2	GPCR. TGF beta inducible	AF008914	2.45	2.85
THRA. Thyroid hormone receptor alpha	Nuclear receptor	U09504	2.15	2.60
ROR alpha. RAR-related orphan receptor alpha	Orphan nuclear receptor.	U53228	1.55	2.60
PLAUR-plasminogen activator, urokinase receptor	Localizing and promoting plasmin formation	X62700	2.06	2.50
NRP1. Neuropilin 1, VEGF165 receptor	Cell surface glycoprotein. Receptor for Semaphorin III	D50086	2.13	1.27

MERTK. C-mer proto-oncogene tyrosine kinase	Receptor tyrosine kinase	U21301	0.48	1.50
GHR. Growth hormone receptor	Cytokine family of receptors	M31680	0.46	0.98
IL4R. Interleukin-4 receptor (secreted form)	Cytokine family of receptors	M27960	0.49	0.44
VEGFR2. Vascular endothelial growth factor receptor 2	Receptor tyrosine kinase	D88689	0.89	0.43
Notch homologue 1		Z11886	0.54	0.43
NTRK3. Neurotrophin-3 receptor	Receptor tyrosine phosphatase	AF035400	0.76	0.43
LRP1. Low density lipoprotein receptor related protein 1	Alpha-2-macroglobulin receptor	X67469	0.83	0.38
OSMR. Oncostatin M receptor beta subunit	Cytokine family of receptors	AB015978	0.78	0.37
sFRP-2. Secreted frizzled related protein 2	Modulator of Wnt signaling	U88567	0.94	0.34
PDGFR alpha. Platelet derived growth factor receptor, alpha	Receptor tyrosine kinase	M57683	0.48	0.33
Notch gene homologue 3		X74760	0.37	0.27
LRP5. Low density lipoprotein receptor-related protein 5	Component of the Wnt receptor complex.	AF064984	0.25	0.32
Or6 gene.	Olfactory receptor of the OR37 subfamily. GPCR	AJ133430	0.20	1.00
MPT	Receptor tyrosine phosphatase	D13903	0.50	0.20
RDC1. Chemokine orphan receptor 1	GPCR	AF000236	0.41	0.13
PTGER1. Prostaglandin E receptor 1 (subtype EP1)	GPCR	Y07611	1.60	0.11

Cytoskeleton

Name	Description	Accession number	Relative expression level	
			Day 1	Day 3
KIFC1	Kinesin family motor protein C1	D49544	1.62	3.08
Alpha-tubulin	Testis-specific	M13443	1.63	2.91
KIF4	Kinesin family motor protein 4	D12646	1.49	2.82
Vinculin	Cytoplasmic face of adhesion plaques	L18880	0.83	2.76

Dia2 Diaphanous-related formin	RhoA binding protein	AF094519	1.40	2.50
NDPP-1	Microfilament assembly and cell motility	D10727	2.36	0.82
LIMK	LIM-domain containing, Ser/Thr protein kinase. Activated by Rho small GTPase. Regulates actin cytoskeletal reorganization	U15159	2.35	2.20
Eg5	Kinesin-related mitotic motor protein	AJ223293	1.28	2.11
Myosin X		AJ249706	1.23	2.02
ARHGAP5	Rho GTPase activating protein 5	U67160	0.48	1.00
LSP1. Lymphocyte-specific protein 1	Cytoskeleton-associated protein	D49691	0.47	0.80
VIL2. Villin	Connections of major cytoskeletal structures to the plasma membrane	X60671	0.39	0.55

Signaling

Name	Description	Accession number	Relative expression level	
			day 1 +/-	day 3 +/-
CAPN6. Calpain 6.	Ca2+-dependent intracellular non-lysosomal protease believed to participate in signal transduction	Y12582	1.00	4.05
IFI204. Interferon activated gene 204	Probably mediates growth inhibitory effect of interferon	M31419	2.50	1.00
ADAM10. A disintegrin and metalloprotease domain 10	Cell-cell and cell-matrix interaction. Controls proteolytic processing of Notch	AF011379	0.87	2.32
GRB. Growth factor receptor bound protein 10	Plays a functional role in insulin and IGF-I signaling	AF022072	0.64	2.08
SOCS1	Cytokine inducible negative regulator of Janus kinase/STAT signaling	U88325	2.04	1.21
plp92	Immediate early gene. Activated by different MAPK pathways by growth factors or environmental stress.	M59821	0.94	2.03
CAPN5. Calpain 5	Ca2+-dependent intracellular non-lysosomal protease believed to participate in signal transduction	Y10656	0.74	0.49
ITPR2. Inositol trisphosphate receptor type 2	Coupled to Ca2+ channels. Release of Ca2+ from intracellular stores	AF031127	0.74	0.48
PIK3R1	p85, regulatory subunit of PI3K	U50413	0.46	0.88
KSR1	PK. Positive modulator of Ras-dependent signaling	U43585	0.45	1.00

CalDAG-GEFI	Rap guanine nucleotide exchange factor. Rap-stimulate or inhibit MAPK pathway	U78171	0.44	1.00
SOCS-2. Suppressor of cytokine signalling-2	Jak/STAT signaling inhibitor. Suppresses GH and IGF-1	U88327	0.41	0.40
Ndr1. N-myc downstream regulated 1	Involved in stress responses, hormone responses, cell growth and differentiation	U52073	0.52	0.39
TPD52L1. Tumor protein D52-like 1	Calcium-mediated signal transduction and cell proliferation	AF004428	0.77	0.35
ADCY7. Adenylate cyclase 7	Membrane-bound, Ca+2 inhibitable adenylate cyclase	U12919	0.59	0.34
NEDD9 (cas l)	Docking protein. Plays a central role for tyrosine-kinase-based signaling related to cell adhesion	AF009366	1.30	0.34
ACK2	Non-receptor protein tyrosine kinase. Associated with integrin signaling. Cdc42-regulated	AF037260	0.59	0.31
GBP2	Guanylate binding protein 2, interferon-inducible	AJ007970	0.31	0.27
GOA-alpha	Guanine nucleotide-binding protein Go, alpha subunit	M36777	0.50	0.23
R-Ras	Small GTPase. Affects cell adhesion by maintaining integrin activity	M21019	0.19	0.53
WISP-2	Wnt-1-induced signaling protein. Connective tissue growth factors family	AF100778	0.21	0.16
NOV. Nephroblastoma overexpressed gene	Insulin-like growth factor binding protein family	Y09257	0.19	0.15
CRABP2. Cellular retinoic acid binding protein II.	Regulates the access of retinoic acid to the nuclear retinoic acid receptors.	M35523	0.14	0.34
PLCbeta1	Phospholipase C beta1. Activated by G alpha-q and G alpha-11	U85714	0.13	4.21
TRAF5	Tumor necrosis factor (TNF) receptor-associated factor 5	D78141	1.00	0.11

Kinases

Name	Description	Accession number	Relative expression level	
			Day 1	Day 3
Serine/threonine kinase 5	Possible role in cell growth	D21099	0.08	5.65
esk/TTK kinase	Ser/Thr/Tyr (dual-specificity) kinase, in rapidly proliferating cell lines	M86377	1.53	3.16
LYN	Tyr protein kinase, belongs to Src family	M57696	1.69	2.91
EEF2K	Eukaryotic elongation factor-2 kinase	U93848	1.00	2.85

BUB1	Mitotic checkpoint Ser/Thr-protein kinase	AF002823	1.45	2.84
Mst1. Ste20-like protein kinase	Stress-responsive protein kinase	U28726	1.15	2.77
FRK (fyn-related kinase)	Tyr-protein kinase, related to Src family.	Z48757	2.70	0.54
STK6	Ser/Thr kinase 6 –mitotic centosomal protein kinase	U80932	1.75	2.62
PLK Polo-like kinase homologue	Ser/Thr kinase, required for mitosis	U01063	1.94	2.37
LIMK	Ser/Thr protein kinase. Regulates actin cytoskeletal reorganization	U15159	2.35	2.20
Calcium/calmodulin-dependent protein kinase II delta	Ser/Thr protein kinase	AF059029	0.40	2.35
NEK2	Ser/Thr kinase. Mitotic regulation	AF013166	1.43	2.29
RIPK1	Ser/Thr protein kinase. Required for TNFR1 activation of NF-kappa b	U25995	0.67	2.10
STK18	Ser/Thr kinase 18. Polo-family of mitotic regulators	L29480	1.47	2.01
MEK1	Dual specificity mitogen-activated protein kinase kinase 1	L02526	1.07	0.49
DMPK. Dystrophia myotonica kinase	Important role in Ca2+ homeostasis and signal transduction system. Mutations in myotonic dystrophy	Z38015	0.35	0.99

Transcription Factors

Name	Description	Accession number	Relative expression level	
			Day 1	Day 3
TCF7	Target of Wnt signaling.	X61385	0.93	8.25
Hey1	bHLH transcription factor. Notch target gene	AJ243895	8.05	3.30
Mybl2	Cell cycle progression	X70472	7.90	5.30
LEF1. Lymphoid enhancer binding factor 1	Activation of Wnt responsive genes	D16503	1.00	7.85
junB	BMP target gene	U20735	5.00	6.05
LEF1. Lymphoid enhancer binding factor 1	Activation of Wnt responsive genes	D16503	1.00	4.85
Oct-11	POU domain, class 2	Z18537	3.25	4.30
Msx2	Homeobox domain, msh-like 2. Increased in early OB differentiation	X59252	4.20	1.30

Id1. Inhibitor of DNA binding 1	HLH transcriptional inhibitor. BMP-2 inducible	M31885	4.00	0.67
Id2. Inhibitor of DNA binding 2	HLH transcriptional inhibitor. BMP-2 inducible	AF077861	3.97	2.01
EGR1/Krox 24. Early growth response 1	Up-regulated in OB after mechanical loading; Egr1-/- mice: induced production of M-CSF, stimulation of osteoclasts	M28845	0.33	3.62
HOXC9	Homeobox transcription factor	X55318	3.55	1.54
Fosl1(Fra-1) Fos-like antigen 1	AP-1 component. Involved in OB and osteoclast differentiation	AF017128	3.40	1.00
Id3. Inhibitor of DNA binding 3	HLH transcriptional inhibitor. BMP-2 inducible	M60523	3.01	3.25
Dlx2	Homeobox. Craniofacial patterning and morphogenesis, development of ventral forebrain	M80540	3.16	1.39
HFH-11	Fork head/ winged-helix transcription factor	Y11245	1.63	2.89
CLOCK	bHLH transcription factor	AF000998	2.71	0.87
SMAD6	TGF-beta/BMP signaling pathway, inhibitory molecule	AF010133	2.63	1.36
Zac1	Zn finger protein. Promotes cell cycle arrest and apoptosis; act as a positive or negative transcriptional cofactor for nuclear receptors	X95503	1.45	2.57
Hlx	Homeobox transcription factor. Involved in embryogenesis and hematopoiesis	X58250	2.56	1.09
BRCA1	Zn finger superclass. Transcriptional regulation of p21 in response to DNA damage	U32446	2.25	2.42
EGR2/Krox20. Early growth response 2	Zn finger superclass. Transition from the promyelinating to the myelinating stage of Schwann cell development	M24377	0.70	2.27
SMAD7	TGF-beta/BMP signaling pathway, inhibitory molecule	AF015260	2.25	0.88
MafG	Transcriptional modulator. Interaction with various basic-leucine zipper-type transcription factors.	AB009693	1.00	2.20
TIEG	TGF-beta inducible. Transcriptional repressor	AF064088	2.18	1.91
Mrg1a	TALE homeobox protein subfamily	U68383	1.00	2.10
c-myc	BHLH transcription factor	L00039	0.96	2.09
SMAD1	BMP signaling pathway, stimulatory molecule	U58992	1.38	2.08
PML	Zn-finger nuclear protein. Tumor suppressor, regulates p53 response to oncogenic signals	U33626	2.07	1.10

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TCF4. Transcription factor 4	HMG box. Activation of Wnt responsive genes	AF107298	1.04	2.03
AT motif binding factor 1	Multiple homeobox domain, zinc finger transcription factor	D26046	0.50	0.56
HMX3	Homeobox transcription factor	X75330	1.00	0.48
FHL1. Four and a half LIM domains protein 1	Involvement in muscle development or hypertrophy	U41739	0.51	0.48
Oct1	POU2F1 (POU domain, class 2, transcription factor 1). Transcription factor for small nuclear RNA and histoneH2b genes.	X68363	0.51	0.47
FHL2. Four and a half LIM domains protein 2	Transcriptional coactivator. Transmits Rho signals	AF055889	0.74	0.45
ZFP97	Kruppel family of Zn finger proteins	L20450	0.43	1.13
HIC-1. Hypermethylated in cancer 1	BTB/POZ transcriptional repressors	AJ007511	1.00	0.43
NFI-B2	Member of NFI/CTF family	D90173	0.42	0.93
PBX1	TALE homeobox protein subfamily. Pre B-cell leukemia transcription factor	L27453	0.84	0.35
Six-1.Sine oculis-related homeobox 1	Six homeobox proteins control muscle formation through activation of one of its key regulators, myogenin	X80339	0.46	0.32
Eos	Icarus family of transcription factors. Lymphoid development	AB017615	0.29	1.00
ISGF3G	Interferon dependent	U51992	0.28	0.31
p51/p73L/p63/p40 gene	p53 homologue, modulates p53 function	AB010152	0.34	0.27
AP-2	Developmental regulation	U17291	0.32	0.26
FOG. Friend of GATA-1	Cofactor for transcription factor GATA-1 in erythroid and megakaryocytic differentiation	AF006492	1.53	0.25
Msx1	Homeobox domain, msh-like 1. Early OB differentiation	X14759	1.05	0.25
IRF5 Interferon regulatory factor 5	Helix-turn-helix transcription factor, involved in innate immune response to infection	AF028725	1.79	0.21
Mel-18	Polycomb group of proteins, regulates expression of Homeobox genes	D90085	0.21	1.00
HoxB8	Homeobox transcription factor. Part of a developmental regulatory system	M18399	0.20	0.93
Id4	HLH transcriptional inhibitor	AJ001972	0.49	0.13
ATFx	Leucine-zipper protein. Interact with G-CSF gene promoter element 1-binding protein	AB012276	0.11	0.31
Cell cycle and				

Apoptosis				
Name	Description	Accession number	Relative expression level	
			Day 1	Day 3
Cyclin D1	G1/S transition control	M64403	5.90	2.49
CDC25C	Protein tyrosine phosphatase. Progression of cell cycle	U15562	1.27	3.44
GTSE-1	G2 and S phase expressed protein	AJ222580	1.85	3.11
Caspase 7	Apoptosis-related cysteine protease	U67321	1.84	3.04
Sha1	S-M checkpoint control	AF062378	1.06	2.70
CDCREL-1 homologue	Cell division control	AF033350	2.70	1.49
Survivin -BIRC5	Inhibitor of caspase 3 and 7	AB013819	1.26	2.57
MyD118	Negative growth control	X54149	2.53	1.65
CDC25C	Dosage-dependent inducer in mitotic control	L16926	1.38	2.15
Cyclin A2	Cell cycle control	X75483	1.55	2.11
RIPK1	Ser/Thr protein kinase. Initiates apoptosis.	U25995	0.67	2.10
Ect2	Regulation of cytokinesis	L11316	1.23	2.07
Siah-1	p-53 inducible mediator of cell cycle arrest	Z19580	2.00	1.40
BNIP3L	Nuclear gene encoding mitochondrial protein. Induces apoptosis	AF067395	0.49	0.68
BID	Induces ice-like proteases and apoptosis	U75506	0.33	0.47
GAS2	Growth arrest specific 2	M21828	0.45	0.46
GAS1	Growth suppression. Blocks entry to s . phase	X65128	0.89	0.38
Bcl-2	Suppresses apoptosis	L31532	0.33	0.75
Proliferin	Secreted protein. Stimulate cell proliferation	K03235	1.00	0.29
GAS6	Growth-arrest	X59846	1.00	0.24

DNA Replication				
Name	Description	Accession number	Relative expression level	
			Day 1	Day 3
NASP, nuclear autoantigenic sperm protein	Transporting newly synthesized histones to the nucleus. Testis and sperm-specific.	AF034610	1.71	5.55
Blm	ATP dependent DNA helicase, involved in DNA replication and repair	AB008674	5.50	3.45
IMPDH1: Inosine monophosphate dehydrogenase type I	Rate limiting enzyme in de novo synthesis of guanine nucleotide	U00978	1.61	5.40
CDC45-related protein	Initiation of DNA replication	AF098068	4.40	1.00
HMG2 High mobility group protein 2	Non-histone structural protein of chromatine	X67668	1.56	3.25

CDC46	Initiation of DNA replication	D26090	2.67	3.07
CHAFA	Chromatin assembly factor-I p150 subunit	AJ132771	2.35	2.76
ORC1-related protein	Initiation of DNA replication	AJ003133	2.65	1.63
CDC6-related protein	Initiation of DNA replication	AJ223087	2.37	2.53
POLE2- DNA polymerase epsilon small subunit	DNA replication	AF036898	2.50	2.40
POLD2-DNA polymerase delta 2, regulatory subunit	DNA replication	Z72486	2.42	1.23
Ribonucleotide reductase M2 subunit	Provides precursors for DNA synthesis	M14223	2.23	2.09
MCMD4	Initiation of DNA replication	D26089	2.23	2.23
P1 (S. cerevisiae)	Mcm3 homologue. Initiation of DNA replication	X62154	1.65	2.14
DHOH-dihydroorotate dehydrogenase	Mitochondrial inner membrane, fourth step in pyrimidine biosynthesis	AF029667	1.70	2.06
NP95	Involved in DNA replication-linked nuclear events	D87908	1.87	2.02
Dihydrofolate reductase	Required for purine synthesis	J00388	2.00	1.65
HMG4	Non-histone structural protein of chromatine	AF022465	1.25	2.00
HMGIC	Non-histone structural protein of chromatine	X99915	0.67	0.44
MtPolB mitochondrial DNA polymerase accessory subunit	Nuclear gene encoding mitochondrial protein, replication of mitochondrial genome	AF006072	0.32	0.74
H2B and H2A histone	Structural protein of chromatine	X05862	0.30	0.67
H1 histone	Structural protein of chromatine	J03482	1.00	0.16

Others

Name	Description	Accession number	Relative expression level	
			Day 1	Day 3
COX2. Cyclooxigenase2	Prostaglandin-endoperoxide synthase 2. Mitogen- and cytokine- inducible form	M88242	4.55	1.25
Plasminogen				
tPA	Plasminogen activator, tissue	J03520	0.73	7.02
PAUR1	Plasminogen activator, urokinase receptor	X62700	2.06	2.50
Nexin-I	Inhibitor of urokinase-type plasminogen activator (uPA)	X70296	0.51	0.39
Cholesterol				

HMG-CoA reductase	Control of cholesterol biosynthesis, rate-limiting enzyme in steroid synthesis	M62766	2.14	11.65
SC5D. sterol-C5-desaturase	Cholesterol biosynthesis		1.07	3.00
DHCR7. 7-dehydrocholesterol reductase	Cholesterol biosynthesis	AF057368	1.93	2.85
LAL. Lysosomal acid lipase	Intracellular control of cholesterol and trglyceride catabolism	Z31689	2.09	0.50
Immunophilins				
FKBP5. FK506 binding protein 5	Immunophilin, intracellular receptor for immunosuppressive drug FK506. Peptidyl-prolyl cis/trans isomerase	U16959	1.04	7.04
FKBP1b. FK506-binding protein 1b	Immunophilin, intracellular receptor for immunosuppressive drug FK506. Peptidyl-prolyl cis/trans isomerase	AF060872	0.48	0.42

Total RNA was extracted from non-stimulated confluent cells (day 0) and from cells treated with osteogenic stimulus (GP/AA/BMP-2) (+) or GP alone (-) for 1 and 3 days. RNA samples were analyzed by GeneChip microarray and by NPGN and Expressionist software. The numbers represent fold regulation by osteogenic stimulus, relative to time-matched controls. The genes were considered regulated if their expression changed >2-fold, as compared to the time-matched controls. Red - up-regulated genes; black-down-regulated genes. Within each functional group, the genes were sorted according to the level of regulation, from the strongest stimulation to the strongest inhibition, including both days 1 and 3. In rare cases where the gene was both up-and down-regulated at different times, higher regulation level was taken as a sorting criterium.

(b)   *Table 2.   Expression profiles of genes related to osteoblast differentiation and support of osteoclastogenesis.*

BMP-2 signaling pathway				
Name	Description	Accession number	Relative expression level	
			Day 1	Day 3
Gremlin	Secreted. BMP antagonist	AF045801	0.12	0.15
SMAD1	BMP signaling pathway	U58992	1.38	2.08
SMAD6	TGF-beta/BMP signaling pathway, inhibitory molecule	AF010133	2.63	1.36
SMAD7	TGF-beta/BMP signaling pathway, inhibitory molecule	AF015260	2.25	0.88
BMP-2 target genes				
Dlx2	Homeobox. Craniofacial patterning and morphogenesis	M80540	3.16	1.39
Id1. Inhibitor of DNA binding 1	HLH transcriptional inhibitor. BMP-2 inducible	M31885	4.00	0.67
Id2. Inhibitor of DNA binding 2	HLH transcriptional inhibitor. BMP-2 inducible	AF077861	3.97	2.01
Id3. Inhibitor of DNA binding 3	HLH transcriptional inhibitor. BMP-2 inducible	M60523	3.01	3.25
Id4	HLH transcriptional inhibitor	AJ001972	0.49	0.13
JunB	BMP target gene	U20735	5.00	6.05
Related to osteoblast differentiation				
Alkaline phosphatase	OB differentiation marker	J02980	8.10	43.75
BMP-4	TGF-beta superfamily. Stimulates OB differentiation	L47480	0.44	0.77
Bone sialoprotein	Integrin binding sialoprotein	L20232	1.00	12.85
Fosl1 (Fra-1) Fos-like antigen 1	AP-1 component. Involved in OB and OC differentiation	AF017128	3.40	1.00
LEPR. Leptin receptor	Cytokine family of receptors	U42467	1.00	4.95
LRP5. Low density lipoprotein receptor-related protein 5	Component of the Wnt receptor complex.	AF064984	0.25	0.32

Msx1	Homeobox domain, msh-like 1. Early phase of OB differentiation	X14759	1.05	0.25
Msx2	Homeobox domain, msh-like 2. Early phase of OB differentiation	X59252	4.20	1.30
Osteocalcin	Bone gamma carboxyglutamate protein 1	L24431	1.00	10.20
PTHR-Parathyroid hormone receptor	GPCR	X78936	2.55	33.85
ROR alpha. RAR-related orphan receptor alpha	Orphan nuclear receptor	U53228	1.55	2.60
Stimulation of osteoclastogenesis				
M-CSF	Macrophage colony-stimulating factor. Stimulates osteoclastogenesis.	M21952	0.74	0.41
EGR1/Krox 24. Early growth response 1	Up-regulated in OB after mechanical loading; Egr1-/- mice: induced production of MCSF, stimulation of osteoclast.	M28845	0.33	3.62
OPG. Osteoprotegerin	Tumor necrosis factor receptor superfamily, member 11b. Inhibits OC genesis	U94331	1.27	3.84
Parathyroid hormone-like peptide (PTHRP)	Supports osteoclastogenesis, osteolytic bone metastases and cartilage-matrix degradation during endochondral bone formation	M60057	0.23	0.18
Small inducible cytokine A7	Chemotactic factor for monocytes	X70058	0.74	0.45

Selected data from Table 1, grouped according to their known role in regulating bone homeostasis, osteoblasts and osteoclasts. Red – up-regulated genes; black – down-regulated genes.

(c)   *Table 3.   Expression profiles of components of TGFβ signaling pathway.*

Ligands

Name	Description	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
TGF-beta1	TGF-beta family	AJ009862	37	34	155	82	292
TGF-beta2.	TGF-beta family	X57413	74	78	74	97	42
TGF-beta3	TGF-beta family	M32745	685	700	1467	502	562

Receptors

Name	Description	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
TGF-beta receptor I	TGF-beta receptor	L15436	74.5	43.5	59.5	28.4	55.3
TGF-beta receptor II	TGF-beta receptor	D32072	A	A	A	A	A

Effectors

Name	Description	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
SMAD2	TGF-beta activated SMAD	Not on the chip					
SMAD 3	TGF-beta activated SMAD	AB008192	A	A	A	A	A
SMAD4	Common SMAD	Not on the chip					
SMAD6	Inhibitory SMAD	AF010133	270	276	727	204	277
SMAD7	Inhibitory SMAD	AF015260	114	107	241	104	92

Regulated target genes

Name	Description	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
TIEG	TGF beta inducible. Transcriptional repressor	AF064088	41	66	144	46	88
Tenascin C	Extracellular matrix glycoprotein. Secreted by OB. Stimulate OB differentiation. TGF-beta Induced	X56304	666	883	2238	721	2038
TGFBI. Kerato-epithelin	Extracellular adhesion molecule, binds to Col 1,2 and 4. TGF beta Induced	L19932	114	131	457	79	162
SSTR2. somatostatin receptor type 2	GPCR. TGF beta Induced	AF008914	20	20	49	20	57

Selected data from Table 1, grouped according to their known role in TGF-β signaling. The numbers represent absolute expression levels on microarrays. Day 1, 3+ - samples treated with osteogenic stimulus, Day 1, 3- - time-matched controls. A - absent (signal <20). Red – up-regulated genes; black – down-regulated genes.

(d)   Table 4.   Expression profiles of components of Wnt signaling pathway.

Ligands						
Name	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
Wnt1	M11943	A	A	46	A	A
Wnt2	Not on the chip					
Wnt2B	AF070988	A	A	A	A	A
Wnt3	M32502	A	A	A	A	A
Wnt3A	X56842	A	A	A	A	A
Wnt 4	M89797	A	A	A	A	A
Wnt 5A	M89798	A	A	A	A	A
Wnt 5B	M89799	40	A	34	26	39
Wnt6	M89800	24	23	40	27	104
Wnt 7A	M89801	A	A	A	A	A
Wnt7B	AI852662	A	A	A	A	A
Wnt8B	Not on the chip					
Wnt8D	Z68889	A	A	A	A	A
Wnt10A	U61969	28	A	32	A	136
Wnt 10B	U61970	79	71	76	42	65
Wnt 11	X70800	A	A	A	A	A
Receptors						
Name	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
Frizzled homologue 1	AF054623	77.9	62.6	65.8	A	A
Frizzled homologue 2	AW123618	894.5	809.4	655.1	786.7	630.4
Frizzled homologue 3	U43205	A	A	A	A	A
Frizzled homologue 4	U43317	A	A	A	A	A
Frizzled homologue 5	Not on the chip					
Frizzled homologue 6	U43319	A	A	A	A	A
Frizzled homologue 7	U43320	A	A	A	A	A
Frizzled homologue 8	U43321	150.7	102.1	96.7	130.5	79.2
Frizzled homologue 9	Y17709	A	A	26	45	30
Secreted frizzled-related sequence proteins (sFRPs)						
sFRP1	U88566	A	A	A	A	A
sFRP2	U88567	3698	3772.998	3539.999	3689.001	1266
sFRP4	AF117709	A	A	A	A	A
Low density lipoprotein receptors						
Name	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
LDLR	Z19521	266.4	352.8	587.9	381.1	548.5
LRP1	X67469	871.5	783.5	653.2	1167.2	447.6
LRP2	AW259788	A	A	A	A	A
LRP4	AB013874	A	A	A	A	A
LRP5	AF064984	48	80	A	62	M
LRP6	AF074265	54.8	22.8	A	35.8	21.3

Receptor antagonists						
Name	**Accession number**	**Day 0**	**Day 1-**	**Day 1+**	**Day 3-**	**Day 3+**
Dickkopf 1	AF030433	A	A	A	A	A
Dickkopf 2	AJ243963	A	A	A	A	A
Dickkopf 3	AJ243964	1078.7	605.5	517.8	609.7	327.9
Effects						
Name	**Accession number**	**Day 0**	**Day 1-**	**Day 1+**	**Day 3-**	**Day 3+**
Beta catenin	M90364	2311.6	2312.3	1530.1	2044.6	2201.1
Axin	AF009011	264.4	349.2	353.1	406.8	390.6
APC	M88127	40.2	48.1	50.9	32.7	31.5
Glycogen synthase kinase 3	Not on the chip					
Lymphoid enhancer binding factor 1 (LEF1)	D16503	A	A	A	A	97
TCF4. Transcription factor 4	AF107298	156	136	142	60	122
Regulated target genes						
Name	**Accession number**	**Day 0**	**Day 1-**	**Day 1+**	**Day 3-**	**Day 3+**
T-cell transcription factor 7 (TCF7)	X61385	28	29	27	A	165

Selected data from Table 1, grouped according to their known role in Wnt signaling. The numbers represent absolute expression levels on microarrays. Day 1, 3+ - samples treated with osteogenic stimulus, Day 1, 3- - time-matched controls. A - absent (signal <20). M - marginal signal.

(e)　　Table 5.　　Expression profiles of components of Notch signaling pathway.

Ligands

Name	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
Delta-like homologue 1	X80903	A	A	A	A	A
Delta-like homologue 2	Not on the chip					
Delta-like homologue 3	AF068865	A	A	A	A	A
Jagged 1	Not on the chip					
Jagged 2	Y14331	A	A	A	A	A

Receptors

Name	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
Notch homologue 1	Z11886	64	91	49	157	67
Notch homologue 2	D32210	A	A	A	A	A
Notch homologue 3	X74760	227.2	135.2	50.1	249.2	66.6
Notch homologue 4	Not on the chip					

Notch regulation

Name	Accession number	day 0	day 1-	day 1+	day 3-	day 3+
ADAM10	AF011379	123	75	65	31	72
Furring	Not on the chip					
Presenilin 1	L42177	350	340	352	344	346
Presenilin 2	U57325	40	28	21	61	34
Lunatic fringe homologue	AF015768	A	A	64.1	A	36.2
Radical fringe homologue	AF015770	328.2	321.5	374.8	292.6	384.7
Manic fringe homologue	Not on the chip					

Effects

Name	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
RBPJk	X17459	374	213	94	327	113

Direct target genes

Name	Accession number	Day 0	Day 1-	Day 1+	Day 3-	Day 3+
HES-1	D16464	135	123	126	72	134
HES-2	AB009967	A	A	A	A	A
HES-3	D32200	A	A	A	A	A
HES-4	Not on the chip					
HES-5	D32132	A	A	A	A	A
HES-6	AW048812	65	48	43	63	56
Hey-1	AJ243895	A	A	161	A	66
Hey-2	Not on the chip					
Hey-L	Not on the chip					

Selected data from Table 1, grouped according to their known role in TGF-β signaling. The numbers represent absolute expression levels on microarrays. Day 1, 3+ - samples treated with osteogenic stimulus, Day 1, 3- - time-matched controls. A - absent (signal <20).